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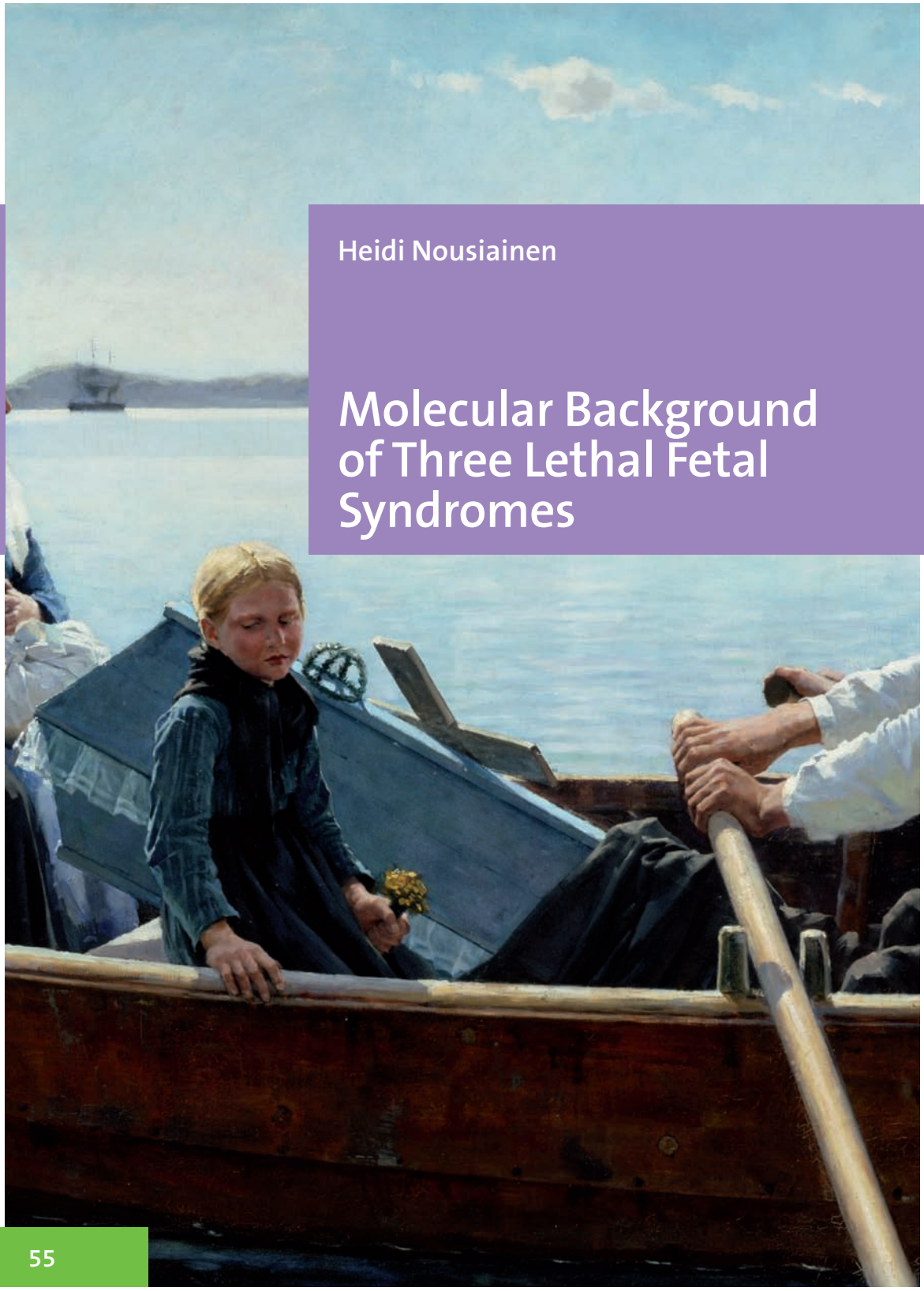

RESEARCH

Heidi Nousiainen

Molecular Background of Three Lethal Fetal Syndromes

Research 55

Heidi Nousiainen

Molecular Background of Three Lethal Fetal Syndromes

ACADEMIC DISSERTATION

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"This time it had been magic. And it didn't stop being magic just because you found out how it was done."

-Terry Pratchett (The Wee Free Men)

To my family

Abstract

Heidi Nousiainen. Molecular Background of Three Lethal Fetal Syndromes. National Institute for Health and Welfare (THL), Research 55/2011. 142 pages. Helsinki, Finland 2011.
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This study identified the molecular defects underlying three lethal fetal syndromes. Lethal Congenital Contracture Syndrome 1 (LCCS1, MIM 253310) and Lethal Arthrogryposis with Anterior Horn Cell Disease (LAAHD, MIM 611890) are fetal motor neuron diseases. They affect the nerve cells that control voluntary muscle movement, and eventually result in severe atrophy of spinal cord motor neurons and fetal immobility. Both LCCS1 and LAAHD are caused by mutations in the *GLE1* gene, which encodes for a multifunctional protein involved in posttranscriptional mRNA processing. LCCS2 and LCCS3, two syndromes that are clinically similar to LCCS1, are caused by defective proteins involved in the synthesis of inositol hexakisphosphate (IP_6), an essential cofactor of GLE1. This suggests a common mechanism behind these fetal motor neuron diseases, and along with accumulating evidence from genetic studies of more late-onset motor neuron diseases such as Spinal muscular atrophy (SMA) and Amyotrophic lateral sclerosis (ALS), implicates mRNA processing as a common mechanism in motor neuron disease pathogenesis.

We also studied *gle1*$^{-/-}$ zebrafish in order to investigate whether they would be a good model for studying the pathogenesis of LCCS1 and LAAHD. Mutant zebrafish exhibit cell death in their central nervous system at two days post fertilization, and the distribution of mRNA within the cells of mutant zebrafish differs from controls, encouraging further studies.

The third lethal fetal syndrome is described in this study for the first time. Cocoon syndrome (MIM 613630) was discovered in a Finnish family with two affected individuals. Its hallmarks are the encasement of the limbs under the skin, and severe craniofacial abnormalities, including the lack of skull bones. We showed that Cocoon syndrome is caused by a mutation in the gene encoding the conserved helix-loop-helix ubiquitous kinase CHUK, also known as IκB kinase α (IKKα). The mutation results in the complete lack of CHUK protein expression. CHUK is a subunit of the IκB kinase enzyme that inhibits NF-κB transcription factors, but in addition, it has an essential, independent role in controlling keratinocyte differentiation, as well as informing morphogenetic events such as limb and skeletal patterning. CHUK also acts as a tumor suppressor, and is frequently inactivated in cancer. This study has brought significant new information about the molecular background of these three lethal fetal syndromes, as well as provided knowledge about the prerequisites of normal human development.

Keywords: LCCS1, LAAHD, GLE1, motor neuron, Cocoon syndrome, CHUK, fetal development, developmental disorder, Finnish disease heritage

Tiivistelmä

Heidi Nousiainen. Molecular Background of Three Lethal Fetal Syndromes [Kolmen letaalin sikiöaikaisen oireyhtymän molekyylitausta]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 55/2011. 142 sivua. Helsinki 2011.
ISBN 978-952-245-453-9 (printed), ISBN 978-952-245-454-6 (pdf)

Tässä väitöskirjatyössä tunnistettiin kolmen sikiökautena kuolemaan johtavan sairauden aiheuttavat perimän muutokset. Hervan tauti eli Letaali synnynnäinen kontraktuuraoireyhtymä (LCCS1, MIM 253310) ja Vuopalan tauti eli Letaali selkäytimen etusarvitauti (LAAHD, MIM 611890) ovat liikehermosairauksia, jotka johtavat kuolemaan jo kohdussa tai pian syntymän jälkeen. Väitöskirjatyössä osoitettiin, että sekä Hervan tauti että Vuopalan tauti aiheutuvat *GLE1*-geenin virheistä. Solubiologisten tutkimusten perusteella tiedetään, GLE1-proteiinilla on solussa keskeinen tehtävä lähetti-RNA:n prosessoinnissa. Viime vuosina tehdyt geenilöydökset viittaavat siihen, että lähetti-RNA:n prosessointiin liittyvät häiriöt ovat taustalla myös muissa liikehermosoluja rappeuttavissa sairauksissa. Väitöskirjatyössä tutkittiin myös *gle1*-geenin suhteen mutantteja seeprakaloja ja todettiin, että kaloilla on kahden vuorokauden iässä solukuolemaa keskushermoston alueella ja lähetti-RNA:n jakauma solussa on epänormaali.

Lisäksi tässä työssä kuvattiin ensimmäistä kertaa kokonaan uusi oireyhtymä, jossa sikiön raajat ovat koteloituneet ihon sisälle ja pään sekä kasvojen alueen rakenne on epämuodostunut. Oireyhtymä kuvattiin yhdessä suomalaisessa perheessä, jossa oli kaksi sairasta sikiötä, ja sen aiheuttava perimän muutos tunnistettiin *CHUK*-geenistä. Uuden oireyhtymän nimeksi annettiin Cocoon-oireyhtymä (MIM 613630) (engl. cocoon = kotelo, suojakalvo).

Väitöskirjatyö on tuottanut merkittävää uutta tietoa sikiöaikaisten kuolemaan johtavien sairauksien syntymekanismeista sekä myös normaalin kehityksen edellytyksistä.

Avainsanat: LCCS1, LAAHD, GLE1, liikehermosolu, Cocoon-oireyhtymä, CHUK, sikiönkehitys, kehityshäiriö, suomalainen tautiperintö

Table of contents

Abstract 7
Tiivistelmä 8
List of original publications 11
Author's contribution to the publications 12
Abbreviations 13

1 INTRODUCTION 15

2 REVIEW OF THE LITERATURE 16
2.1 The Finnish Disease Heritage 16

2.2 Fetal motor neuron disease 21
2.2.1 Motor neuron disease 21
2.2.2 Fetal akinesia deformation sequence (FADS) 27
2.2.3 Lethal Congenital Contracture Syndrome 1 (LCCS1) 27
2.2.4 Lethal arthrogryposis with anterior horn cell disease (LAAHD) 29
2.2.5 LCCS2 and LCCS3 30
2.2.5.1 ERBB3 and PIP5K1C are associated with phosphoinositide synthesis 32
2.2.5.2 Erbb3 -/- mice 35
2.2.5.3 *Pip5k1c* $^{-/-}$ mice 35
2.3 Molecular characterization of diseases 37
2.3.1 Characterization of disease genes through functional studies 37
2.3.2 Genetic resources 38
2.3.3 Positional cloning 38
2.3.4 Homozygosity mapping 39
2.3.5 Other genomic methods 40
2.3.6 Mutations 41
2.4 Human fetal development 42
2.4.1 Neuronal development 43
2.4.2 Development of craniofacial structures 47
2.4.3 Development of the epidermis and cutaneous structures 48
2.4.4 Errors in development 50
2.5 Animal models 51
2.5.1 The Zebrafish as a model of neurodegeneration 52

3 AIMS OF THE STUDY 53

4 SUBJECTS, MATERIALS AND METHODS 54
4.1 Subjects and samples 54
4.1.1 Ethical aspects 54
4.2 Methods 54
4.2.1 Methods used in original publications 54
4.2.2 Materials and methods used in unpublished studies 55

5 RESULTS AND DISCUSSION 57
5.1 Identification of the LCCS1 disease gene (I) 57
5.1.1 Screening LAAHD families for mutations in *GLE1* 62
5.1.2 The effect of the mutations on the GLE1 protein 62
5.1.3 Screening additional patients for mutations in *GLE1* (unpublished data) 63
5.2 *GLE1* function 64
5.2.1 Overexpression of mutant and wild type GLE1 in HeLa-cells 65
5.2.2 *In situ* hybridization 65
5.2.3 LCCS1, LCCS, and LCCS3 disease genes are linked by a common pathway 66
5.2.4 Posttranscriptional RNA processing 67
5.2.5 RNA processing and motor neuron disease 68
5.2.6 *gle1* $^{-/-}$ zebrafish (unpublished data) 69
5.3 Fetal encasement malformation (II) 73
5.3.1 Clinical description of the cases 73
5.3.2 Genealogical investigations 74
5.3.3 Genome-wide gene expression array analysis 75
5.3.4 Analysis of the *CHUK* gene and CHUK protein 76
5.3.5 CHUK and NF-κB 78
5.3.6 *Chuk* $^{-/-}$ mice 79
5.3.7 Pathway analysis of the gene expression array data 80
5.3.8 MMP-14 80
5.3.9 CHUK and NF-κB signalling in the development of ectodermally derived structures 81
5.3.10 CHUK, keratinocyte differentiation and skin cancer 82
5.3.11 Cocoon syndrome 83

6 CONCLUSIONS AND FUTURE PROSPECTS 84

7 ACKNOWLEDGEMENTS 85

Original publications

List of original publications

I **Heidi O. Nousiainen**, Marjo Kestilä, Niklas Pakkasjärvi, Heli Honkala, Satu Kuure, Jonna Tallila, Katri Vuopala, Jaakko Ignatius, Riitta Herva, Leena Peltonen. Mutations in mRNA export mediator *GLE1* result in a fetal motoneuron disease. *Nature Genetics* 2008 Feb;40(2):155-7.

II Jenni Lahtela*, **Heidi O. Nousiainen***, Vedran Stefanovic, Jonna Tallila, Heli Viskari, Riitta Karikoski, Massimiliano Gentile, Carola Saloranta, Teppo Varilo, Riitta Salonen, Marjo Kestilä. Mutant *CHUK* and severe fetal encasement malformation. *New England Journal of Medicine* 2010 Oct;363(17):1631-1637

III In addition, this thesis includes unpublished data.

*the authors contributed equally to the work

Author's contribution to the publications

I HN contributed to all of the experiments, wrote the manuscript, and edited the manuscript in collaboration with the other authors.

II HN participated in the study design. HN also contributed to the genealogic, sequencing, and protein studies. HN wrote the manuscript, and edited the manuscript in collaboration with the other authors and journal editors.

Abbreviations

ALS	Amyotrophic lateral sclerosis
AO	Acridine orange
BMP	Bone morphogenic protein
cDNA	complementary DNA
CHUK	conserved helix-loop-helix ubiquitous kinase
CNS	central nervous system
CNV	copy number variation
DNA	deoxyribonucleic acid
EJC	Exon junction complex
ES cell	embryonic stem cell
FADS	fetal akinesia deformation sequence
FDH	Finnish disease heritage
FGF	Fibroblast growth factor
HGP	Human genome project
HLS	Hydrolethalus syndrome
hnRNP	heterogenous ribonucleoprotein particle
HSP	Hereditary spastic paraplegia
IBD	identical by descent
IP_6	inositol hexakisphosphate
IKKα	Inhibitor of kappa B (IκB) kinase α; also known as IKK1 and CHUK
LAAHD	Lethal arthrogryposis with anterior horn cell disease
LCCS1	Lethal congenital contracture syndrome 1
LCCS2	Lethal congenital contracture syndrome 2
LCCS3	Lethal congenital contracture syndrome 3
LMN	lower motor neuron
LOD	logarithm of odds
Mb	megabase
MIM	Mendelian inheritance in man
MND	motor neuron disease

MKS	Meckel syndrome
mRNP	messenger ribonucleoprotein particle
NGF	Nerve growth factor
NFκB	nuclear factor kappaB
NMD	nonsense mediated mRNA decay
kb	kilobase
LD	linkage disequilibrium
mRNA	messenger RNA
PCR	polymerase chain reaction
PIP	phosphoinositide
PLC	phospholipase C
PLS	Primary lateral sclerosis
PTC	premature termination codon
PTEN	phosphatase and tensin homolog
PTU	1-phenyl 2-thiourea
RNA	ribonucleic acid
SBMA	Spinal bulbar muscular atrophy
SCC	squamous cell carcinoma
SMA	Spinal muscular atrophy
SNP	single nucleotide polymorphism
SOD1	superoxidase dismutase 1
UMN	upper motor neuron

1 Introduction

The molecular background of lethal fetal syndromes is an interesting and important research topic for numerous reasons. First and foremost, parents that have lost a child, or often several children to such a disease, benefit from any information that can be provided to them about the condition in their family. Once a causative mutation for a syndrome is found, DNA testing can immediately be applied in its diagnostics. In addition, deciphering the cellular processes that are disturbed in these developmental disorders also provide invaluable information about the prerequisites of normal human development, and ultimately, increase our knowledge of biology in general. Finally, knowledge gained from the study of these rare syndromes often benefits the research of more common diseases that are linked to the same organ system, cell type, or molecular pathway.

This research focused on three lethal fetal syndromes; two of which are well-established clinical entities, and one, which we describe here for the first time. Lethal Congenital Contracture Syndrome 1 (LCCS1, MIM 253310) and Lethal Arthrogryposis with Anterior Horn Cell Disease (LAAHD, MIM 611890) are fetal motoneuron diseases. They affect the nerve cells that control voluntary muscle movement, and eventually result in severe atrophy of spinal cord motoneurons and fetal immobility. Both are inherited in an autosomal recessive manner, and are more common in Finland than elsewhere in the world. They represent the most extreme end in a spectrum of Motor Neuron Diseases (MNDs), an etiologically heterogeneous group of disorders perpetually under intensive research effort. The third condition studied here is a fetal encasement malformation found in one family of Finnish origin. The first pregnancy of the family ended in miscarriage, and multiple fetal malformations were seen in two consecutive pregnancies, which were terminated. The most prominent features of the affected fetuses were the encasement of the limbs under the skin, and severe craniofacial abnormalities, including the lack of skull bones.

The aim of this study was to identify the molecular defects underlying LCCS1 and LAAHD, and to describe in detail the novel fetal encasement syndrome, and investigate its molecular background.

2 Review of the literature

2.1 The Finnish Disease Heritage

The Finnish Disease Heritage (FDH), a concept first introduced by Perheentupa (Perheentupa 1972) and Norio (Norio et al. 1973), refers to a group of inherited conditions that are, in proportion to population size, more common in Finland than elsewhere in the world. Currently 36 individual diseases fulfill this definition. 32 of these are inherited in an autosomal recessive manner, two are autosomal dominant, and two are X-chromosomal (Norio 2003c). The fact that these hereditary diseases are found in Finland, while others, such as phenylketonuria and cystic fibrosis are not, does not imply that Finns are less healthy than other nations; it merely reflects the uniqueness of the Finnish population (Norio 2003a). It is possible that the number of FDH diseases will increase in the future; not by the means of Finns getting sicker, but through careful evaluation of novel clinical entities, that might eventually fit the definition of a FDH disease. The acceptance of a novel syndrome into the Finnish disease heritage is usually done through mutual agreement of the FDH research community. As a general rule, a minimum of ten individual families has to be known for a given disease (Norio. 2000d).

The roots of the FDH lie in the Finnish population history. It has been estimated that Finland was first inhabited some 10,000 years ago, after the Late Glacial Maximum (Nunez 1987, Norio 2003b). Due to geographical and cultural isolation, migration has been exiguous. During the course of history, the population has been shaped by both local and national bottlenecks, and has eventually grown to its current size of over five million individuals. Up to the 16th century, the habitation was situated mainly along the coastline (currently referred to as the *early settlement region*), until the political actions of King Gustavus of Wasa induced an internal migration towards the then uninhabited central, eastern, and northern parts of the country. These areas now constitute the so-called *late settlement region*. The majority of these late settlers originated from southern Savo (Figure 1), and established local communities, which became subisolates as they remained separate from each other due to long distances and low population density (De la Chapelle 1993, Peltonen et al. 1999, Norio 2003b). This settlement history is still reflected in the genetic architechture of Finns, not only in terms of disease, but also in the general population (Jakkula et al. 2008, Salmela et al. 2008). The population of each subisolate carried their own unique set of genes, including disease mutations. Consanguineous marriages were unavoidable in these regional subisolates, although

the relations between individuals usually dated back several generations, and the couples themselves were unaware of them (Norio 2003a). This led to clustering of certain diseases in certain parts of the country. During the 20th century the population distribution of Finland underwent changes, and settlement concentrated increasingly in urban communities. Thus cases of the FDH no longer manifest solely in particular regions, but are found throughout the country. However, the birthplaces of the greatgrandparents of the cases still show regional clustering in many of the diseases, reflecting the historical migration of the Finnish population (Varilo 1999, Norio 2003c). Albeit being overrepresented in relation to population size on a global scale, the FDH diseases are rare also in Finland, with incidences of the diseases varying between 1:8,000 and 1:100,000. This means that approximately six to eight cases suffering from a given disease are born each year. Although the disease spectrum of FDH covers several branches of medicine, it is most distinctly visible in pediatrics. Many of the diseases result in severe handicap and are a heavy burden to the patient and family, but some diseases can be treated simply and effectively once a correct diagnosis is established (Norio 2003a).

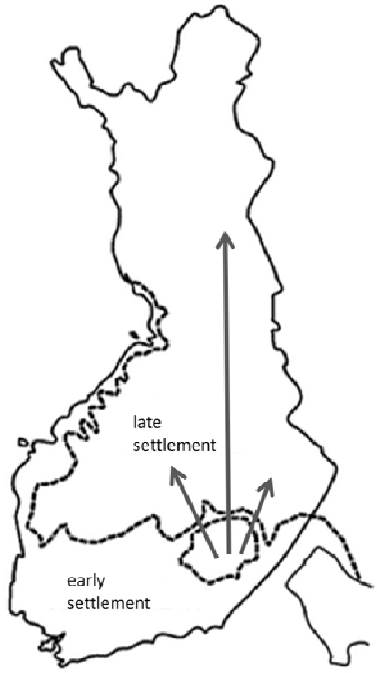


Figure 1. A model for the internal population migration of Finland that occurred in the 16th century.

The molecular genetics of the FDH is well studied, and the genetic defect for all of the diseases, except for one, has now been published (Table 1). In most diseases, one founder mutation accounts for >90% of the cases. However, in some diseases the founder mutation is present only in 70-80% of the cases and additional mutations of the same gene are found in varying frequencies (Sankila et al. 1992, Kestilä et al. 1998, Huopaniemi et al. 1999). Even locus heterogeneity within the Finnish population has been observed, namely in Meckel syndrome, in which mutations in three different loci have been identified in Finnish cases, and additional loci are likely to be discovered (Kyttälä et al. 2006, Tallila et al. 2008, Tallila et al. 2009). A database of the Finnish diseases and mutations can be found online (www.findis.org).

Of the 32 recessive diseases of the FDH, five are invariably lethal during the embryonic period or soon after birth. Meckel syndrome (MKS, MIM 249000) and Hydrolethalus syndrome (HLS, MIM 236680), which are the most common of the embryonically lethal FDHs with incidences of 1:9,000 and 1:20,000, respectively, are both characterized by severe malformations of multiple organs, including the digits and central nervous system. In addition, large, polycystic kidneys are seen in Meckel syndrome. The genetic defect has been identified in both syndromes, enabling the use of DNA testing in diagnostics, as well as further studies on the molecular background of these disorders (Mee et al. 2005, Kyttälä et al. 2006, Tallila et al. 2008). Studies suggest that at least Meckel syndrome, or possibly both MKS and HLS, are linked to ciliary dysfunction (Dammermann et al. 2009, Gerdes et al. 2009). In addition, studies on the HYLS1 protein, which is defective in HLS, implicate altered lipid metabolism, cell cycle regulation, and signalling in the pathogenesis of HLS (Honkala et al. 2009). Another neonatally lethal disease of the FDH is GRACILE syndrome (MIM 603358), which is characterized by growth retardation, aminoaciduria, cholestasis, iron overload, and lactacidosis. GRACILE syndrome is caused by the defective mitochondrial inner membrane protein BCS1L, although there is some question whether the disease pathogenesis has to do with mitochondrial dysfunction, or another, currently uncharacterized cellular function on BCS1L, putatively associated with iron metabolism (Visapää et al. 2002). The incidence of GRACILE is 1:47,000. The two remaining lethal disorders, Lethal congenital contracture syndrome and Lethal arthrogyposis with anterior horn cell disease, will be discussed in detail in section 2.3.

Table 1. Diseases of the Finnish disease heritage. A reference is given for the first publication(s) of a Finnish mutation for each disease, except PEHO, for which the causative mutation has not yet been published, and for which a reference of the decription of the phenotype is given.

Name of the disease	MIM	Symbol	Reference
Recessive:			
Aspartylglucosaminuria	208400	AGU	(Ikonen et al. 1991)
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy	240300	APECED	(Nagamine et al. 1997, The Finnish German APECED Consortium 1997)
Cartilage-hair hypoplasia	250250	CHH	(Ridanpää et al. 2001)
Cohen syndrome	216550	COH1	(Kolehmainen et al. 2003)
Congenital chloride diarrhea	214700	CLD	(Höglund et al. 1996)
Congenital lactase deficiency	223000	–	(Kuokkanen et al. 2006)
Congenital nephrosis	256300	CNF	(Kestilä et al. 1998)
Cornea plana congenita	217300	CNA2	(Pellegata et al. 2000)
Diastrophic dysplasia	222600	DTD	(Hästbacka et al. 1994)
Finnish variant of late infantile neuronal ceroid lipofuscinosis	256731	vLINCL	(Savukoski et al. 1998)
FSH-resistant ovaries	233300	FSH-RO	(Aittomäki et al. 1995)
GRACILE syndrome	603358	GRACILE	(Visapää et al. 2002)
Gyrate atrophy of choroid and retina	258870	GA	(Mitchell et al. 1988)
Hydrolethalus syndrome	236680	HLS	(Mee et al. 2005)
Infantile neuronal ceroid lipofuscinosis	256730	INCL	(Vesa et al. 1995)
Infantile onset spinocerebellar ataxia	271245	IOSCA	(Nikali et al. 2005)
Lethal arthrogryposis with anterior horn cell disease	611890	LAAHD	(Original publication I)
Lethal congenital contracture syndrome	253310	LCCS	(Original publication I)
Lysinuric protein intolerance	222700	LPI	(Borsani et al. 1999, Torrents et al. 1999)
Meckel syndrome	249000	MKS	(Kyttälä et al. 2006)
Mulibrey nanism	253250	MUL	(Avela et al. 2000)
Muscle-eye-brain disease	253280	MEB	(Diesen et al. 2004)
Neuronal ceroid lipofuscinosis, juvenile	204200	JNCL	(The International Batten Disease Consortium 1995)
Nonketotic hyperglycinemia	605899	NKH	(Kure et al. 1992)
PEHO syndrome	260565	PEHO	(Salonen et al. 1991)
Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy	221770	PLOSL	(Paloneva et al. 2000)

Progressive epilepsy with mental retardation	600143	EPMR	(Ranta et al. 1999)
Progressive myoclonus epilepsy	254800	EPM1	(Pennacchio et al. 1996, Virtaneva et al. 1997)
RAPADILINO syndrome	266280	RAPADILINO	(Siitonen et al. 2003)
Selective intestinal malabsorption of vitamin B12	261100	MGA1	(Aminoff et al. 1999)
Sialic acid storage disease	604369	SD	(Verheijen et al. 1999)
Usher syndrome, type III	276902	USH3	(Joensuu et al. 2001)
Dominant:			
Familial amyloidosis, Finnish type	105120	FAF	(Levy et al. 1990, Maury et al. 1990)
Tibial muscular dystrophy	600334	TMD	(Hackman et al. 2002)
X-chromosomal:			
Choroideremia	303100	CHM	(Sankila et al. 1992)
Retinoschisis	312700	RS	(The Retinoschisis Consortium 1998)

The research of the molecular genetics of rare syndromes, such as diseases of the FDH, can contribute substantially to the research of more common diseases as well. For example, nephrin, a key structural protein of the glomerular podocyte in the kidney, was discovered via molecular genetic analysis of patients with Finnish type Congenital nephrotic syndrome (CNF) (Kestilä et al. 1998). CNF is rare outside Finland, but the interest in nephrin is global, since it is an essential structural protein of the kidney and is also involved in important signalling processes. Thus, studies of the nephrin protein contribute to understanding the physiology of the kidney in health and disease. A particular area of focus is now understanding the role of nephrin in the podocyte insulin response and diabetic nephropathy, which is the most common cause of endstage renal failure in the Western world and, along with the current 'epidemic' of obesity and type II diabetes, a rapidly increasing burden on both morbidity and health economics (Welsh and Saleem 2010).

2.2 Fetal motor neuron disease

2.2.1 Motor neuron disease

Motor neuron diseases (MNDs) are an etiologically heterogeneous group of disorders that affect the nerve cells controlling voluntary muscle movement. MNDs include diseases such as the childhood-onset Spinal muscular atrophy (SMA) and the late-onset Amyotrophic lateral sclerosis (ALS). Table 2 lists some motor neuron diseases and their clinical characteristics. (Dion et al. 2009)

Table 2. Classification and clinical characteristics of motor neuron diseases, modified from (Dion et al. 2009).

Disease	Age of onset	Prevalence	Motor neuron involvement	Clinical features
ALS	Between 45 and 60 years old	4-6/1000,000	UMNs and LMNs	Progressive muscle weakness, atrophy and spasticity
HSP	From early childhood to 70 years old	3-10/100,000	UMNs	Progressive spasticity in the lower limbs
PLS	Between 35 and 66 years old	1/10,000,000	UMNs	Spinal and bulbar spasticity
SMA	Between 6 and 18 months old for type I, II and III; between 15 and 50 years for type IV	1/6,000-10,000	LMNs	Symmetrical muscle weakness and atrophy
SBMA	Between 30 and 50 years old	1-9/100,000	LMNs	Slowly progressive limb and bulbar muscle weakness with fasciculations, muscle atrophy and gynecomastia
LCCS	Fetal	1/25,000	UMNs and LMNs	Early fetal hydrops and akinesia, degeneration of anterior horn motor neurons and extreme skeletal muscle atrophy
LAAHD	Fetal	>1/100,000	UMNs and LMNs	Fetal akinesia, arhtogryposis and motor neuron loss.

ALS, amyotrophic lateral sclerosis; HSP, hereditary spastic paraplegia; LAAHD, lethal arthrogryposis with anterior horn cell disease; LCCS, lethal congenital contracture syndrome; LMN, lower motor neuron; PLS, primary lateral sclerosis; SBMA, spinal bulbar muscular atrophy; SMA, spinal muscular atrophy; UMN, upper motor neuron.

Approximately 10% of Amyotrophic lateral sclerosis is familial, usually inherited in an autosomal dominant manner, although recessive and X-linked forms also exist (Bento-Abreu 2010) (Table 3). However, 90% of ALS cases are sporadic with no apparent family history. Most of the knowledge of the pathogenesis of ALS comes from the study of the rare genetic forms (Bento-Abreu 2010). Superoxidase dismutase 1 (SOD1) mutations are the most common cause of familial ALS. SOD1 mutations are seen in 20% of familial ALS patients, thus explaining 2% of all ALS cases. SOD1 is a ubiquitously expressed enzyme that catalyzes the conversion of superoxide free radicals to hydrogen peroxide. It is active as a homodimer. Missense mutations affecting almost every single one of the 153 amino acid residues comprising the SOD1 protein have been found, usually inherited in an autosomal dominant form. One particular mutation, D90A, which is common in Finnish ALS patients, is found throughout the world and usually inherited in an autosomal dominant manner, but in some Finnish and Swedish patients it follows an autosomal recessive mode of inheritance (Andersen et al. 1996, Parton et al. 2002). The disease-associated haplotype surrounding the recessive and dominant D90A mutations is different, suggesting a presence of a modifying genetic factor that could explain this difference between recessive and dominant D90A forms (Parton et al. 2002). Studies on murine models suggest a toxic gain-of-function as the pathogenic mechanism behind motor neuron toxicity induced by mutant SOD1 (Reaume et al. 1996), but despite nearly two decades of intensive research efforts, the mechanism by which mutant SOD1 induces motor neuron degeneration remains incompletely understood. (Bento-Abreu 2010)

Spinal muscular atrophy (SMA) often refers to the most common form of the disease, proximal SMA, caused by loss-of-function mutations in the survival motor neuron 1 (*SMN1*) gene. Proximal SMA results from reduced expression levels of the full-lenght SMN protein. SMN is encoded by the *SMN1* and *SMN2* genes. The majority of SMN protein, however, is derived from the *SMN1* gene. The *SMN2* gene contains a translationally silent C-T transition that alters an exonic splice enhancer motif and results in the exclusion of exon 7 from most *SMN2* transcripts yielding a highly unstable SMN protein that is degraded by the ubiquitin-proteasome system (Burnett et al. 2009). The *SMN* protein is widely expressed in different tissues, and together with Gemin proteins, it forms a complex that is important in the assembly, recycling, and maintenance of small nuclear ribonucleoproteins (snRNPs), components of the spiceosome (Kolb et al. 2007). In addition to proximal SMA caused by *SMN1* mutations, there are multiple forms of SMA that are distinguishable by age of onset and molecular background, summarized in Table 4. (Wee et al. 2010)

In hereditary spastic paraplegias (HSPs), mutations in twenty different genes are currently known. The proteins encoded by these genes, and thus implicated in the pathogenesis of HSPs, can be grouped into three categories according to their cellular functions; membrane traffic -related proteins, mitochondrial proteins, and proteins related to myelination (Blackstone et al. 2011).

Primary lateral sclerosis (PLS) is an autosomal disorder that only affects upper motor neurons in the corticospinal and corticobulbar tracts. Unlike ALS, lower motor neuron involvement in not seen in PLS. However, there are patients who present pure upper motor neuron signs for years but later progress to typical ALS, so there is some clinical overlap with ALS. The PLS locus has been mapped to chromosome 4p (Valdmanis et al. 2008). PLS can also result from ALS2 mutations (Dion et al. 2009).

Table 3. Genetic background of familial ALS, modified from (Bento-Abreu 2010)

Type of familial ALS	Inheritance	Onset	Chr	Gene	Protein function
ALS1	AD (AR)	adult	21q	*SOD1*	Enzyme that catalyses the conversion of superoxide free radicals to hydrogen peroxide
ALS2*	AR	childhood	2q	*ALS2*	Functions as a guanine nucleotide exchange factor for the small GTPase RAB5. Localizes with RAB5 on early endosomal compartments, and functions as a modulator for endosomal dynamics.
ALS3	AD	adult	18q	*?*	
ALS4	AD	childhood/ adolescent	9q	*SETX*	Contains a C-terminal DNA/RNA helicase domain suggesting involvement in both DNA and RNA processing
ALS5	AR	childhood	15q	*?*	
ALS6	AD (AR)	adult	16p	*FUS*	Involved in RNA processing
ALS7	AD	adult	20p	*?*	
ALS8*	AD	adult	20q	*VAPB*	Type IV membrane protein found in plasma membranes and intracellular vesicle membranes
ALS9	AD	adult	14q	*ANG*	Hydrolyzes cellular tRNAs resulting in decreased protein synthesis. Angiogenin mediates the formation of new blood vessels.
ALS10	AD	adult	1p	*TARDBP*	DNA and RNA binding protein
ALS11	AD	adult	6q	*FIG4*	Contains SAC domain that has phosphoinositide phosphatase activity

*ALS2 is allelic with PLS

*ALS8 is allelic with Adult onset proximal SMA

AR = autosomal recessive, AD = autosomal dominant

Table 4. Genetic background of SMA, modified from (Wee et al. 2010)

Type of SMA	Inheritance	Onset	Chr	Gene	Protein function
Proximal SMAs					
Proximal spinal muscular atrophy	AR	infantile/ childhood	5q	*SMN1*	Forms heteromeric complexes with proteins such as SIP1 and GEMIN4, and also interacts with several proteins known to be involved in the biogenesis of snRNPs.
Infantile SMA with arthrogryposis	X-linked	infantile	Xp	*UBA1*	Catalyzes the first step in ubiquitin conjugation to mark cellular proteins for degradation
SMA with pontocerebellar hypoplasia	AR	congenital/ infantile	14q	*VRK1*	Member of the vaccinia-related kinase (VRK) family of serine/threonine protein kinases potentially involved in regulating cell proliferation.
SMA type I phenotype due to mitochondrial dysfunction	AR	infantile/ childhood	22q	*SCO2*	An assembly factor for the multimeric cytochrome c oxidase (COX) protein complex. COX is involved in aerobic ATP production.
Spinal and bulbar muscular atrophy (SBMA)	X-linked	adult	Xq	*Androgen receptor*	Steroid-hormone activated transcription factor
Adult onset proximal SMA*	AD	adult	20q	*VAPB*	Type IV membrane protein found in plasma membranes and intracellular vesicle membranes
Distal SMAs or hereditary motor neuropathies (HMNs)					
HMN 2A	AD	adult	12q	*HSPB8*	Belongs to the superfamily of small heat-shock proteins containing a C-terminal alpha-crystallin domain
HMN 2B	AD (AR)	adult	7q	*HSPB1*	Stress resistance and actin organization
HMN 2C	AD	adult	5q	*HSPB3*	Muscle-specific small heat shock protein

HMN 4	AR	childhood	1p	*PLEKHG5*	Activates the nuclear factor kappa B (NFKB1) signaling pathway
HMN 5A	AD	adult	7p	*GARS*	Glycyl-tRNA synthetase
HMN 5B	AD	variable	11q	*BSCL2*	ER-localized protein
HMN 7B	AD	adult	2p	*DCTN1*	The largest subunit of dynactin, a macromolecular complex that binds to microtubules and cytoplasmic dynein. Dynactin is involved in ER-to-Golgi transport, the centripetal movement of lysosomes and endosomes, spindle formation, chromosome movement, nuclear positioning, and axonogenesis.
Scalpuloperoneal SMA (SPSMA)	AD	congenital	12q	*TRPV4*	Ca^{2+}-permeable, nonselective cation channel that is thought to be involved in the regulation of systemic osmotic pressure.
Distal SMA, X-linked 3	X-linked	variable	Xq	*ATP7A*	Transmembrane protein that functions in copper transport across membranes.
SMN with respiratory distress 1 (SMARD1, HMN 6)	AR	infantile	11q	*IGHMBP2*	Helicase superfamily member that binds a specific DNA sequence from the immunoglobulin mu chain switch region.

*Adult onset proximal SMA is allelic with ALS8

AR = autosomal recessive, AD = autosomal dominant

2.2.2 Fetal akinesia deformation sequence (FADS)

Pena and Shokeir were the first to describe patients with a lethal syndrome of multiple joint contractures, characteristic facial features including a small chin and low-set ears, and pulmonary hypoplasia (Pena and Shokeir 1974). It was subsequently suggested that the Pena-Shokeir phenotype is not specific enough to delineate one syndrome, but rather a general, secondary phenotype resulting from decreased or absent fetal movement *in utero* (Moessinger 1983, Hall 1986, Hageman et al. 1987), and is therefore currently referred to as the Fetal Akinesia Deformation Sequence (FADS). FADS can be caused by extrinsic factors, such as a maternal tumor, oligohydramnion, or myasthenia gravis, or intrinsic factors, such as fetal muscle-, nerve- or connective tissue disease (Spranger et al. 1982).

2.2.3 Lethal Congenital Contracture Syndrome 1 (LCCS1)

Lethal congenital contracture syndrome 1 (LCCS1, MIM 253310) is an autosomally recessively inherited fetal motoneuron disease. The disease was first described by Dr. Riitta Herva, a pathologist working at the Oulu University Hospital, and is also referred to as Herva disease (Herva et al. 1985). LCCS1 was previously called LCCS, but in 2007 Narkis and colleagues published the causative genes for two syndromes that they called LCCS2 and LCCS3 (Narkis et al. 2007a, Narkis et al. 2007b) (see section 2.2.5.), and thus the name of LCCS was also amended as LCCS1 in 2008 (Original publication I). LCCS1 can be detected relatively early on in the pregnancy; using modern ultrasonogram equipment a diagnosis can be made already during the 11^{th}-12^{th} gestational week. The LCCS1 fetus is small for gestational age and completely immobile with flexion contractures of the elbows, wrists, and hips, and hyperextension of the knees. Other clinical characteristics include pterygia of the elbows and the neck, pulmonary hypoplasia, and typical facial features including eyes that are wide apart, low set ears, and a small chin (micrognathia). In addition to these clinical features consistent with the FADS phenotype, severe hydrops of the fetus is also seen. In the case of LCCS1, the immobility and associated FADS phenotype is caused by the extreme atrophy of spinal cord motoneurons, which can be verified at autopsy (Herva et al. 1988). Also extreme atrophy of skeletal muscles is seen. However, other internal organs, including the brain, appear macroscopically normal. A histological cross section of the spinal cord is needed in order to establish a correct diagnosis.

The LCCS1 locus was mapped to chromosome 9q34 by linkage in 1998. The inital scan using five affected individuals pointed out four chromosomal loci which were identical by descent in all of the affected individuals, but the analysis of a denser marker set in ten families with a total of 20 affected individuals excluded all of these except the region in chromosome 9. The multipoint linkage analysis yielded a

maximum LOD score of 6.5, which peaked between the markers D9S1825 and D9S1830 (Mäkelä-Bengs et al. 1998). One core haplotype segregating with the disease was observed in the LCCS1 families. By analysis of multiallelic markers in LCCS1 family material the critical chromosomal region was further defined between markers D9S1827 and D9S752 based on recombinations in the shared ancestral haplotype. This was done at a time when the genetic map of the region was not yet completed. Sequencing of 18 of the genes within proximity of the region revealed no pathogenic mutations (Pakkasjärvi 2005b). Upon the completion of the Human Genome Project (see section 2.4.1) the genetic map of the LCCS1 locus was finalized, and thus the exact defining of the region as well as systematic analysis of all the regional candidate genes was facilitated.

To further investigate the molecular background of LCCS1, the RNA expression profiles of the spinal cords of LCCS1 fetuses and age-matched controls were compared using the Affymetrix HG-U133A expression arrays. Differentially expressed genes included a total of 34 downregulated transcripts and three upregulated transcripts (Pakkasjärvi et al. 2005a). Grouping of differentially expressed genes according to biological process revealed nine categories that were significantly different in LCCS1 fetuses and controls. These were: neurogenesis, synaptic transmission, transmission of nerve impulse, nerve ensheathment, neuronal maturation, central nervous system development, organogenesis, morphogenesis, and cholesterol biosynthesis. OLIG2, a transcription factor that controls motor neuron differentiation in the ventral spinal cord, and ERBB3, a protein that is crucial for the development of Schwann cells (see section 2.2.5.2), were among the transcripts downregulated in LCCS1 fetuses. Based on these observations, it was suggested that motor neuron degeneration in LCCS1 could be due to oligodendrocyte dysfunction. Also the transcription factor NKX2.2 showed marginal downregulation in LCCS1 spinal cords. In addition, marginal upregulation of PAX6 and GLI2 was observed. (Pakkasjärvi et al. 2005a) OLIG2, NKX2.2, PAX6, and GLI2 are all involved in the development of motor neurons from the ventral neural tube (see section 2.4.1).

Neural precursor cells from LCCS1 fetuses and age-matched controls were cultured *in vitro* to investigate their ability to differentiate into distinct neuronal subtypes. The LCCS1 cells were able to differentiate into oligodendrocytes, astrocytes, and motor neurons, expressing the appropriate neuronal markers, suggesting that the atrophy of motor neurons in LCCS1 patients is not due to defects in neuronal differentiation, but rather defects in cell survival (Pakkasjärvi et al. 2007). No significant differences in the amount of apoptotic or necrotic cells were observed between the LCCS1 and control cell cultures. However, the global transcription

profiles of the LCCS1 cells and control cells differed from each other. The EGFR transcript was among the genes upregulated in LCCS1 cells. EGFR levels are known to determine progenitor cell proliferation and differentiation, potentially suggesting increased proliferation of the LCCS1 cells. Indeed, the LCCS1 cells appeared denser in culture, but statistical testing revealed no significant difference between proliferative activity of LCCS1 and control cells. It has to be noted that control cells and LCCS1 cells were harvested on separate occasions, and although care was taken to ensure consistency in the harvesting of cells, factors such as the difficulty to assess the exact time of death of the fetus from which the cells were harvested may account for differences in their respective abilities to thrive in culture.

2.2.4 Lethal arthrogryposis with anterior horn cell disease (LAAHD)

Lethal arthrogryposis with anterion horn cell disease (LAAHD, MIM 611890) was first described by Dr. Katri Vuopala, and is commonly referred to as the Vuopala disease. The clinical findings in LAAHD are very similar to LCCS1, only slightly less severe. Fetal movement can be seen in the ultrasonogram investigation in the beginning of the second trimester of the pregnancy, but movements decrease during the course of the pregnancy. Contrary to LCCS1, no fetal hydrops is seen, but polyhydramnion has been reported in many of the pregnancies. The fetuses have malpositions in the extremities; the wrists and ankles are turned inwards. Diagnosis by ultrasound examination is possible in some cases. A report from 1995 describes eleven LAAHD families; in these, 53% of the affected fetuses were born at term and 40% prematurely. The mean duration of pregnancy was 34 weeks. 33% of the fetuses were small for gestational age. Three infants were stillborn, five died within an hour from birth, six lived for a few days, and one infant survived for twenty days (Vuopala et al. 1995). The longest survival for a LAAHD patient has been two months (Dr. Herva, personal communication). The LAAHD patients have hypoplastic lungs, and the primary cause of death after birth is respiratory insufficiency. Analysis of skeletal muscle of ten LAAHD cases showed variation in pathological findings; minor changes were seen in four infants, while in the remaining six infants, more distinct groups of atrophic fibers and hypertrophic type 1 fibers were seen (Vuopala et al. 1995). Analysis of a distal muscle sample from one affected infant showed severe neurogenic atrophy. The gross anatomy of the brain was normal, and myelination was normal with respect to gestational age. The motor neurons of the anterior horn of the spinal cord were degenerated and diminished in number.

Figure 2. Left: A LCCS1 fetus (23 weeks) with typical malpositions of the arms and legs, joint contractures and micrognathia. Right: A baby with LAAHD (38 weeks) displaying typical malpositions of the wrists and ankles.

2.2.5 LCCS2 and LCCS3

In 2003, Landau and colleagues reported 23 patients with a syndrome of congenital contractures from a large, inbred Israeli-Bedouin kindred. In two of these cases, the pregnancies ended prematurely at 23 and 26 weeks, respectively, but the rest were carried to term. No fetal hydrops was observed. The newborns were small for gestational age and displayed multiple joint contractures along with severe muscle atrophy of the lower limbs, but lacked pterygia. In 12 cases, an enlarged urinary bladder was observed. Five cases had hydronephrosis and cystic changes of the kidneys. Of the 23 cases, one pregnancy was terminated, three infants were stillborn, 11 died shortly after birth, four newborns survived up to two weeks, one to one month, and one to one year of age. Two girls, aged 12 and 13 years, were still alive at the time of publication of the article. An autopsy was performed on two patients, and atrophy of the anterior horn of the spinal cord was reported in one of them. Muscle biopsies performed on three cases suggested neurogenic muscle atrophy. (Landau et al. 2003) Despite the fact that two patients were still alive at 12 and 13 years of age, respectively, the syndrome was defined as Lethal congenital contracture syndrome type 2 (LCCS2, MIM 607598) (Narkis et al. 2004).

Linkage to the Spinal Muscular Atrophy (SMA) locus on chromosome 5q and the LCCS1 locus on chromosome 9q34 were excluded (Landau et al. 2003). The LCCS2 locus was assigned to a 6.4 Mb interval on chromosome 12q13 by homozygosity mapping using DNA samples from 10 affected and 44 unaffected members of the consanguineous Bedouin tribe (Narkis et al. 2004). The critical chromosomal region was further narrowed down to 4.6 Mb, and sequencing of the regional candidate genes in affected individuals and controls revealed that a frameshift-inducing intronic mutation in the *ERBB3* gene (NM_001982) segregated with the disease (Narkis et al. 2007b). *ERBB3* encodes for an epidermal growth factor (EGF) receptor ERBB3 (HER3), consisting of a polypeptide of 1342 amino acids, but the mutation predicts a truncated polypeptide of 399 amino acids.

LCCS3 is another fetal akinesia phenotype found in Israeli-Bedouin kindreds. The affected newborns are small for gestational age with multiple joint contractures and muscle atrophy of the lower limbs. Of the nine pregnancies reported, four were terminated on the basis of ultrasonographic findings of fetal akinesia and limb contractures. Two infants were born at 29 gestational weeks, and three at term. All of these infants died shortly after birth. None of the families agreed to post-mortem examination, so there is no information available on the histolopathology of the spinal cord or skeletal muscle (Narkis et al. 2007a). By analyzing the DNA of five affected and 25 unaffected individuals, the LCCS3 disease gene was mapped to a 8.8 Mb interval on chromosome 19p13. Sequencing of 30 out of a total of 120 regional candidate genes suggested a missense mutation in exon 7 of the *PIP5K1C* gene (NM_012398) as being the causative mutation for LCCS3. The mutation (c.757G>A) results in the substitution of aspartic acid to asparagine (p.253D>N) in the PIP5K1C polypeptide (Narkis et al. 2007a). PIP5K1C (PIPKIγ) is a member of the PIP kinase type I (PIPKI) family of enzymes. It phosphorylates the fifth hydroxyl group of phosphatidylinositol-4-phosphate (PI-4-P) to generate phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2). Narkis and colleagues assayed the kinase activity of the mutant and wild type PIP5K1C enzymes *in vitro* by generating recombinant proteins from cDNA obtained from fibroblast cells of LCCS3 patients and controls, respectively. PI-4-P was used as a substrate, and [γ^{32}P]ATP was provided in the reaction mix. Quantification of the labeled end product, PI-4,5-P_2 showed that the mutant protein was only able to generate approximately 1,7% of the amount of PI-4,5-P_2 generated by the wild type protein (Narkis et al. 2007a).

2.2.5.1 ERBB3 and PIP5K1C are associated with phosphoinositide synthesis

Phosphoinositides (PIPs) are small signalling molecules that participate in a variety of cellular processes. They regulate vesicular trafficking, and also modulate lipid distribution and metabolism via lipid transfer proteins. In addition, they regulate ion channels, pumps, and transporters, as well as endocytic and exocytic processes (Balla et al. 2009). The various phosphoinositides are formed by a large number of kinases and phosphatase enzymes. Members of the phospholipase C (PLC) enzyme family cleave the phosphatidyl group of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) resulting in the formation of inositol 1,4,5-trisphosphate (IP_3), which in turn is a precursor for another important group of molecules, the inositol polyphosphates (IPs). The inositol polyphosphates play an important role in nuclear processes such as transcription control, mRNA export, and DNA repair, and are also critical for development and signaling (Tsui and York 2010). As one review sums up the importance of these molecules: ”It might be easier at this time to list the processes that are not regulated by inositides in an eukaryotic cell than those that are clearly inositide dependent” (Balla et al. 2009).

PIP5K1C (PIPKIγ), which is defective in LCCS3 patients, is a member of the PIP kinase type I (PIPKI) family of enzymes. PIP type I kinases synthesize phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) by phosphorylating the fifth hydroxyl group of phosphatidylinositol-4-phosphate (PI-4-P) (Anderson et al. 1999). The three isoforms of type I phosphatidylinosito-4-phosphate-5-kinase (PIP5K1A, PIP5K1B, and PIP5K1C) are encoded by three separate genes. Although they are all capable of synthesizing PIP_2, these enzymes have different primary structures, distinct expression levels in different tissues, and localize within different compartments of cells, which suggests that they are likely to have overlapping, but not identical functions (Wang et al. 2007). PIP5K1A is localized in membrane ruffles (Doughman et al. 2003), PIP5K1B is located near endosomes (Padrón 2003), and PIP5K1C is targeted to focal adhesions and synapses (Wenk et al. 2001, Ling et al. 2002, Di Paolo et al. 2004).

ERBB3 is a member of the ERBB (HER) family of receptors that regulate mammalian cell survival, proliferation, and differentiation in response to growth factors such as epidermal growth factor (EGF) and heregulin (Alroy and Yarden 1997, Burden and Yarden 1997). ERBB3 binds the regulatory p85 subunit of type 1 phosphoinositide 3-kinase (PI3K), thus increasing its kinase activity (Hellyer et al. 1998). Mammals have five distinct p85 isoforms (Vanhaesebroeck et al. 2010), and there is some evidence that different p85 isoforms bind different receptors (Xia and Serrero 1999, Inukai et al. 2001), but it is not specified in the literature which

isoform/isoforms of the p85 subunit were observed to interact with ERBB3 (Holt et al. 1994, Hellyer et al. 1998). PI3K type 1 enzymes phosphorylate the 3-hydroxyl group of the inositol ring of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) generating phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P_3), which, in turn, is then quickly converted by phosphatase enzymes into either PI-3,4-P_2 or back to PI-4,5-P_2 (Figure 3). PI3K type 1 enzymes are comprised of two subunits; a regulatory subunit, and a catalytic subunit. In mammals, there are numerous isoforms of both the regulatory and catalytic subunits encoded by distinct genes, potentially giving rise to a number of different enzyme heterodimer molecules (Franke 2008, Vanhaesebroeck et al. 2010). The different isoforms of these subunits are differentially expressed in cells and tissues (Kok et al. 2009). PI-3,4,5-P_3 and PI-3,4-P_2 coordinate the localization and function of multiple effector proteins, including Serine/Threonine and Tyrosine protein kinases (Such as AKT and BKT, respectively), adaptor proteins (such as GAB2), and regulators of the Rac, Ras, and Arf families of small GTPases (Vanhaesebroeck et al. 2010). PTEN acts as a negative regulator of PI-3,4,5-P_3 levels in the cell, and also functions as a tumor suppressor via the AKT/PKB signalling pathway. PTEN is frequently inactivated in cancer. Phosphoinositides and the enzymes that phosphorylate and dephosphorylate them influence various signalling networks that control cellular processes such as growth and proliferation (Figure 4).

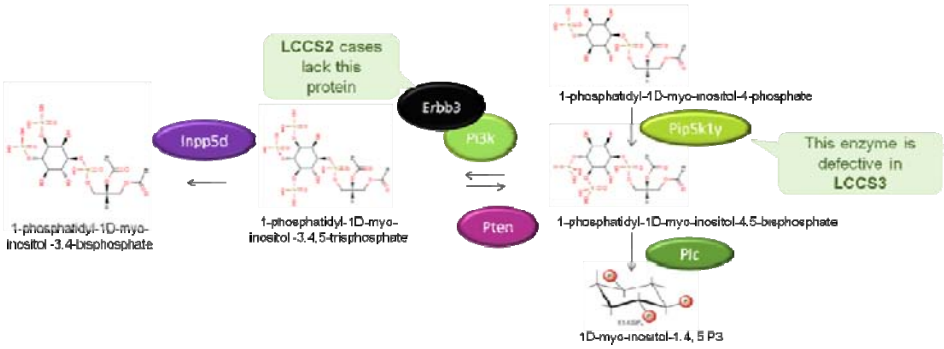


Figure 3. The defective genes in LCCS2 and LCCS3 are both associated with phosphoinositide synthesis.

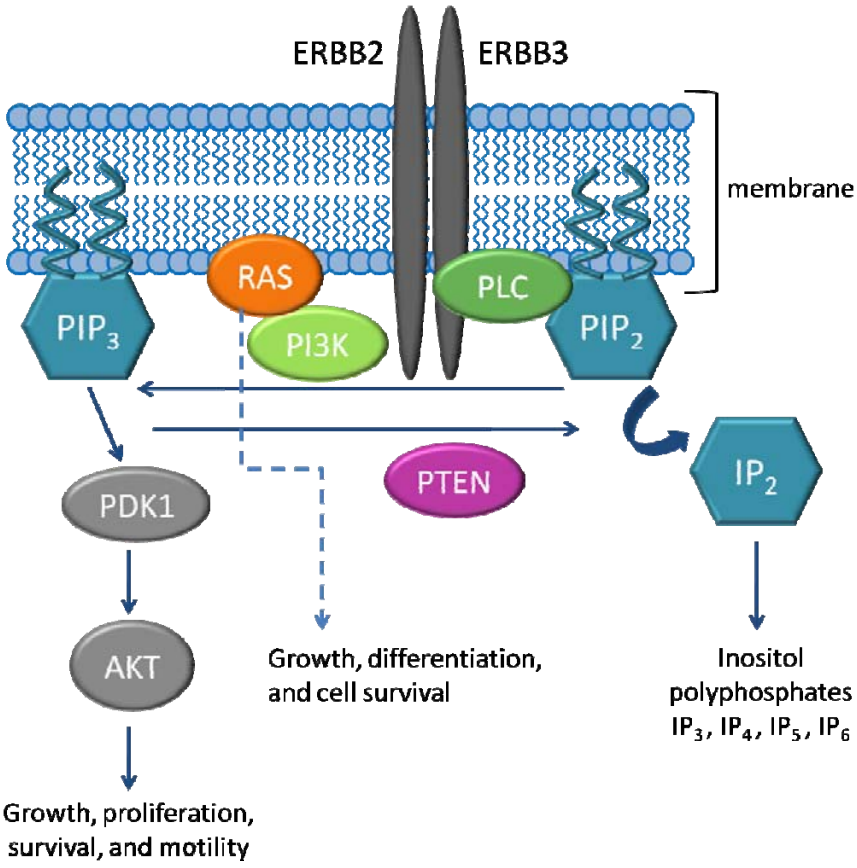


Figure 4. Phosphoinositides PIP_3 and PIP_2 are membrane-bound, whilst the inositol polyphosphates (IPs) are soluble. Phosphoinositides and the enzymes that phosphorylate and dephosphorylate them influence signalling networks that control cellular processes such as growth and proliferation (Balla et al. 2009).

2.2.5.2 Erbb3 -/- mice

Rietmacher and Sonnenberg-Rietmacher generated mice with targeted mutations in *Erbb3*. Heterozygous animals were healthy and fertile, but most homozygous embryos died between E11.5 and E13.5. Surviving embryos developed to term, but newborn pups did not respond to tactile stimulation, were unable to breathe, and died soon after birth. Mutant embryos that were still alive at E16.5, however, did respond to tactile stimulation, although spontaneous movement was reduced. Histological analysis on homozygous pups at E12.5 showed a presence of sensory and motor neurons, but the lack of Schwann-cell precursors that usually accompany them. By E18.5, also the number of sensory and motor neurons was markedly reduced in mutant embryos. The researchers concluded that in homozygous mutant embryos, both sensory and motor neurons start to form, but subsequently degenerate, most likely due to the lack of Schwann cells. (Riethmacher and Sonnenberg-Riethmacher 1997)

2.2.5.3 *Pip5k1c* $^{-/-}$ mice

Two mouse lines with disruptions in the *Pip5k1c* gene have been generated independently by two separate research groups (Di Paolo et al. 2004, Wang et al. 2007). Notably, these two mouse lines have distinctly different phenotypes.

Exons 2–6 of the murine *Pip5k1c* gene, which encode most of the catalytic region, were deleted by homologous recombination by Di Paolo and colleagues to generate *Pip5k1c* $^{-/-}$ animals. The lack of Pip5k1c expression was verified by immunoblotting. Mutant mice are carried to term and are born alive with no obvious anatomical anomalies, but die postnatally within 24h, and can be distinguished from wild type littermates due to impaired motility and the lack of milk in their stomachs (Di Paolo et al. 2004). Analysis of phosphatidylinositol levels in neonatal brain by electrospray ionization mass spectrometry (ESI–MS) revealed a 40% reduction in the PIP_2 levels of *Pip5k1c* $^{-/-}$ animals. ESI-MS is not able to distinguish between PIP_2 isomers (i.e. PI-4,5-P_2, PI-3,5-P_2, PI-3,4-P_2), but since the majority of PIP_2 in the brain is represented by the PI-4,5-P_2 isomer (>95%), it was concluded that PIP5K1C has a major role in mediating PI-4,5-P_2 synthesis in the brain. *Pip5k1c*$^{-/-}$ neurons differentiated *in vitro* similarly to controls based on the analysis of axonal or dendritic processes and of pre- and post-synaptic compartments using specific immunocytochemical markers, but synaptic transmission defects were observed during high-frequency stimulation. The analysis of synaptic vesicle trafficking showed a 20% decrease in the size of the recycling vesicle pool in mutant nerve terminals. Measurement of recycling time, i.e. the time that elapses before dye-loaded endocytic vesicles become available for a new round of exocytosis during

continuous stimulation, revealed that mutant nerve terminals were slower in regaining release competence in comparison to controls, indicating a delay in membrane recycling in the *Pip5k1c*$^{-/-}$ neurons. An assay for real-time measurement of synaptic vesicle exo- and endocytosis indicated that endocytosis was slower in mutant neurons during stimulation in relation to exocytosis. Monitoring of clathrin-mediated endocytosis during stimulation showed that percentage of coated vesicles relative to the total number of vesicles was 3.5-fold lower in *Pipk1c*$^{-/-}$ nerve terminals than in control nerve terminals, and was virtually unchanged from the resting level, suggesting a defect in the clathrin-dependent reformation of synaptic vesicles in mutant animals. (Di Paolo et al. 2004)

Wang and colleagues utilized an ES cell line containing a disruption of the *Pip5k1c* gene generated by β-geo random insertion mutagenesis to create *Pip5k1c* $^{-/-}$ animals. RT-PCR and immunoblot analysis indicated complete loss of the *Pip5k1c* transcript beyond the first exon. Most of the mutant animals die prenatally by embryonic day E11.5, although some survive longer (Wang et al. 2007). The mutant embryos are small in size, have massively dilated pericardial sacs, and anatomical anomalies of the heart. They also exhibit rostral neural tube closure defects (exencelphaly), but closure of the caudal neural tube over the spinal cord is complete. Analysis of mutant embryos revealed that the telancephalic neural folds failed to close, resulting in a disorganized neuroepithelium.

The phenotypic difference between the two *Pip5k1c* knockout animals could be explained by the different genomic background of the two murine strains. Wang and colleagues suggest that the observed differences could be due to modifier genes. An educating example comes from Kumar and colleagues, who observed a surprising phenotype in their knockout animals, and subsequently demonstrated that this phenotype was not due to the targeted deletion of their gene of interest, but a single mutation in another gene, which existed in their mouse embryonic stem cells even before the generation of the knockout mice (Kumar et al. 2004). Since the lack of Pip5k1c expression was verified in both mice, it is a possibility that the difference in phenotype is due to another mutation event. Because of the phenotypic difference of the two *Pip5k1c* knockout mice, the results of the studies have to be interpreted cautiously, and the role of *Pip5k1c* gene in synaptic vesicle trafficking and embryonic development can still not be stated with certainty. However, some results from these studies are pretty straightforward; for example staining for β-gal in heterozygous animals generated by Wang and colleagues showed that Pip5k1c is expressed most prominently in the motor column and in the developing ascending and descending spinal tracts (Wang et al. 2007).

2.3 Molecular characterization of diseases

The first step towards identifying the molecular defect for a disease or a trait is the precise assessment of the phenotype, and careful evaluation of the samples with regard to the phenotype. Following this, numerous different strategies can be utilized to solve the molecular background of the disease. Two main approaches are applicable. One way of thinking is to characterize the defective protein product through its function by application of biological information; the other is to identify the causative gene through its position and sequence within the genome. It is often advantageous to consider both.

2.3.1 Characterization of disease genes through functional studies

Molecular medicine began with the seminal work of Linus Pauling, who recognized a defect in the hemoglobin molecule of patients with sickle cell anemia (Pauling et al. 1949), and Vernon Ingram, who showed that this defect was due to a mutation in the gene encoding hemoglobin beta (Ingram 1957). This was the first time when a single amino acid change in a protein was shown to cause a disorder, and it also demonstrated that if the biochemical basis of a condition is well characterized, its genetic basis can be deduced through the analysis of the defective protein. Other examples of this approach include the identification of the genes underlying hemophilia A (Gitschier et al. 1984) and Aspartylglukosaminuria (Ikonen et al. 1991).

Another way of deciphering the molecular background of a disease is to utilize the information available from existing animal models, for example transgenic mice, whose phenotype resemble the clinical picture of the disease, and for which the genetic defect behind the phenotype is known. An excellent example of this strategy is the identification of a juvenile form of the sensory- and motor neuropathy Charcot-Marie-Tooth disease (CMT4J), in which the disease gene was first mapped in the *pale tremor* mutant mouse, and the sequencing of the homologous gene in human patients resulted in identification of disease mutations (Chow et al. 2007). Sometimes an educated guess can be made about the causative gene, as demonstrated by the example of RAPADILINO syndrome, in which the underlying genetic defect was identified by selection of a candidate gene. Mutations in *RECQL4* were known to cause two syndromes, whose clinical picture was very similar to RAPADILINO. Based on this observation, the *RECQL4* gene was also sequenced in patients with RAPADILINO, and causative mutations were found (Siitonen et al. 2003).

2.3.2 Genetic resources

Genetics provides an excellent tool for tackling the molecular background of diseases. The field of genetics has undergone major advancements during the recent years and continues to develop at an astonishing pace. The Human Genome Project (HGP) was formally initiated in 1990, two of its main objectives being to determine the sequence of the three billion chemical base pairs that make up human DNA and to identify all of the genes contained within human DNA. The approach was to do this by sequencing the complete genome of one individual. In 2001, the working draft of the human genome was published simultaneously by the public International Human Genome Sequencing Consortium (Lander et al. 2001) and the private company Celera Genomics (Venter et al. 2001), and an essentially complete version of the sequence was published a few years later (International Human Genome Sequencing Consortium 2004).

The HGP was followed by the HapMap project, an international effort to catalogue common variation in the human genome between different populations (The International HapMap Consortium 2005). Currently a large amount of effort is put into sequencing the whole genomes or exomes (the RNA-coding regions of a genome) of individuals, the 1000 genomes project (www.1000genomes.org) and the UK10k project (www.uk10k.org) being amongst the most extensive of these undertakings. The HGP, the HapMap project, and the current sequencing enterprises already have, and will also continue to yield an enormous amount of data, which is freely accessible to anyone through public databases. This public data of common genetic variation in humans is useful, for example, when searching for mutations related to disease: comparing patient sequences against it helps filter out the common genetic polymorphisms that are not disease-related. The field of medical genetics has been quick and efficient in exploiting this data with increasingly rapid identification of genes underlying Mendelian traits; the Online Mendelian Inheritance in Man (OMIM) database currently lists 2930 entries of phenotypes for which the molecular basis is known (www.ncbi.nlm.nih.gov/Omim).

2.3.3 Positional cloning

The process of identifying disease genes based on their physical position within the genome is referred to as positional cloning (Collins 1992, Collins 1995). This approach involves the mapping of the disease locus by genomewide genotyping of multiallelic markers followed by linkage analysis. Linkage describes the situation when two genetic loci or alleles are inherited together, i.e. they are *linked*. The aim of a linkage study in disease genetics is to map a disease locus by identifying a genetic marker that segregates with the disease phenotype in a family or multiple families. Linkage is calculated by statistical methods that are based on the testing of

probabilities, and depending on the data available, different methods can be utilized, including singlepoint, multipoint, parametric or non-parametric analysis. Microsatellites are commonly used genetic markers in linkage studies since they are highly variable between individuals, thus being very informative. A linkage study can only be performed if DNA samples are available from an adequate amount of families with a given phenotype. Linkage disequilibrium (LD) refers to a situation in which some combinations of alleles or genetic markers (haplotypes) occur more frequently in a population than would be expected.

Linkage and LD studies have led to the identification of numerous diseases of the FDH. The successful utilization of LD in FDH studies is based on the theory that in any given disease, most of the patients share a single founder mutation inherited from a common ancestor, and this mutation cosegregates with a shared ancestral haplotype. The haplotype, which is derived from an ancestral chromosome, has been restricted over time by recombinations during meioses, and thus the genomic position of the disease-causing mutation can be fine-mapped by monitoring for shared haplotypes in multiple affected families. (Peltonen et al. 1999)

2.3.4 Homozygosity mapping

Homozygosity (or autozygosity) mapping is a useful strategy for identifying disease loci in consanguineous families suffering from a recessive disease (Lander and Botstein 1987). In a child born from a consanguineous marriage, the marker alleles of the region surrounding the disease locus are often homozygous, or identical by descent (IBD), over a distance of several centiMorgans (cM). It has been estimated that approximately 6% of the genome of a child of first cousins is homozygous by descent, but comparing regions of IBD between affected and unaffected children of the same family can help to pinpoint the most likely disease locus. (Lander and Botstein 1987)

Homozygosity mapping has also been successfully used in disease gene identification in an isolated population, where consanguinity dates back several generations. The critical chromosomal region of the disease locus was defined by comparing shared regions of homozygosity between patients from several different families. (Tallila et al. 2008)

2.3.5 Other genomic methods

The current advancement in genomic analysis techniques has brought power and resolution to the study of the genetic background of disorders. Copy number variation (CNV) in particular has been in focus, and current analysis methods allow for the detection of CNVs as small as a few hundred base pairs in size (Conrad et al. 2010). CNVs have been implicated in numerous disorders, including Williams-Beuren syndrome, Charcot-Marie-Tooth type 1 disease, and Neurofibromatosis type 1 (Stankiewicz and Lupski 2010).

Exome sequencing has proven its power in the discovery of disease genes; the cause for Miller syndrome, a rare Mendelian disorder, was identified by massively parallel sequencing of the exomes of four individuals in three different families, combined with filtering to exclude benign and unrelated variants (Ng et al. 2010).

Expression array technology enables the study of transcription profiles of different cells and tissues, allowing the simultaneous examination of the expression of multiple genes and gene networks. Comparisons can be made between the gene expression profiles of cases and controls. Computer programs that process expression array data based on Gene Ontology (Carbon et al. 2009), a controlled vocabulary of terms describing the attributes of genes and gene products, help in classifying differentially expressed genes into groups based on, for example, their localization or function in the cell, or involvement in a known biochemical pathway. This automated grouping of individual genes allows for single molecular events to be perceived in a broader context, and helps in pointing out biological pathways and processes that are represented by the differentially expressed genes.

RNA-seq, or whole transcriptome shotgun sequencing, is a novel method for assaying the mRNA content of a sample, and was developed as an alternative method to gene expression arrays (Wang et al. 2009). ChIP-Seq, or chromatin immunoprecipitation coupled with massively parallel DNA sequencing, allows genome-wide examination of DNA-protein interactions. It can be used to determine how chromatin-associated proteins such as transcription factors, polymerases, transcriptional machinery, structural proteins, protein modifications, and DNA modifications influence the phenotype of an individual. ChIP-Seq can be employed in the study of epigenetic chromatin modifications. (Park 2009)

2.3.6 Mutations

The DNA molecule is susceptible to mutations. Types of mutations include point mutations, insertions, deletions, duplications, inversions, and translocations. A point mutation within the coding region of a gene may be silent, which means that it does not affect the polypeptide encoded by the gene, or a missense mutation, which changes an amino acid within the polypeptide, or a nonsense mutation, which leads to an early stop codon, thus resulting either in a truncated polypeptide, or degradation of the mRNA via nonsense-mediated decay and complete loss of protein expression. Insertions and deletions within the coding region of the gene may cause a shift in the reading frame of the gene or result in aberrant splicing. Intronic mutations may also alter splicing by either creating a novel splice site or abolishing an excisting one. Mutations can also affect regulatory elements of genes, such as transcription factor binding sites, or small noncoding RNAs, such as microRNAs (miRNAs). Larger chromosomal rearrengements and copy number variations (CNVs) may affect one or multiple genes.

Epigenetic modifications of DNA also contribute to human phenotypes. Imprinting, a phenomenon where the expression of certain genes depends on their parental origin, is perhaps the most well known example of this. For example, a deletion in chromosome 15q can result in either Angelman syndrome or Prader-Willi syndrome, depending on the parental origin of the disease chromosome.

A large amount of common variation exists between individuals, but sometimes mutations in DNA can be harmful. In medical genetics, distinguishing between harmful mutations and common variation is not always straightforward. A recent large scale resequencing study shows that, on average, each person carries approximately 250 to 300 loss-of-function variants in annotated genes and 50 to 100 variants previously implicated in inherited disorders (Altshuler et al. 2010). Yet most of us are in good health. Although current advances in DNA sequencing technologies mean that sequencing itself is no longer neither time consuming nor expensive, the storing and analysis of entire human genome sequences, and especially the interpretation of these massive amounts of sequence data have become increasingly challenging. Careful clinical evaluation of patient samples and the use of appropriate control samples, meticulous statistical and bioinformatical analysis, as well as functional validation of disease-associated mutations using cell models or model organisms are needed in order to establish whether a mutation has biological relevance in terms of disease, and should be considered for use in diagnostic testing or the development of drugs and therapies.

2.4 Human fetal development

The development of a human fetus is a complex process guided by both genes and the environment. The formation of a complete organism from a single cell can be divided in three consecutive stages called blastogenesis, organogenesis, and fetogenesis. Blastogenesis takes place during the first four weeks of development and its completion is marked by the closure of the neural tube, followed by organogenesis that lasts until nine weeks of gestation. Fetogenesis lasts from gestational week nine until birth. By this time, the main organs have formed and the fetus continues to grow in size. The formation of organs starts at an early stage, but different organs require different amounts of time to complete their development. The heart is formed at six weeks of gestation, the face and limbs at eight weeks. The eyes start to form during the seventh week of gestation, on the sides of the head, and by the ninth week of gestation, they are situated in the front of the face. The structural development of the brain continues until 28 weeks. Human development requires the precise functioning and coordination of many complex pathways and signaling cascades. Inductive signals between tissues are critical in regulating the development of different organs. (Sariola, et al. 2003, Carlson. 2004)

Blastogenesis begins with fertilization, a process where two cells, the sperm and the egg, fuse together to form the zygote. This is followed by cleavage, during which the zygote undergoes mitotic cell divisions and forms into a blastocyst, while simultaneously migrating from the oviduct into the uterus. Once it has reached the uterus, the blastocyst invaginates into the uterine endometrium and anchors itself into the uterine wall in a process called implantation. Once in place, the trophoblast cells of the blastocyst begin dialogue with the uterine wall initiating the formation of the placenta. Gastrulation, the formation of three germ layers of the embryo, takes place approximately fourteen days after fertilization. These germ layers are the ectoderm, mesoderm, and endoderm. The cells of the ectoderm, the outer layer of the embryo, give rise to the surface layer of the skin (epidermis), as well as the brain and the nervous system. The endoderm, which is the innermost layer of the embryo, generates the lining of the digestive tube and its associated organs, including the lungs. The mesoderm, which lies between the ectoderm and the endoderm, gives rise to the blood, heart, kidneys, gonads, bones, muscles, and connective tissues. Some of the cells of the mesoderm also extend outwards from the embryo. These cells will generate the blood vessels of the umbilical cord that connects the embryo to the placenta. Once the three germ layers are established, their cells interact with one another and rearrange themselves into tissues and organs in a process called organogenesis. The beginning of organogenesis is marked by the formation of the neural tube, which will become the brain and the spinal cord. (Gilbert. 2010)

An important aspect of studying human fetal development is understanding the process of differentiation. During human development a single cell, the fertilized egg, divides and gives rise to hundreds of different cell types. Skin cells, muscle cells, different types of neuronal cells, along with many others, are formed in this process. However, almost every cell of the body contains the same set of genes, so a part of understanding development is determining how the same set of genetic instructions can produce different types of cells. (Gilbert. 2010) This dilemma is mirrored in the study of genetic disease, where often, a defect in an ubiquitously expressed protein results in a tissue-specific phenotype, or a single common mutation is found in patients with a diverse array of clinical symptoms.

The differentiated cells divide, migrate, aggregate, and die, and tissues fold and separate. During organogenesis, certain cells undergo long migrations from their place of origin to their final location. For example, the cells that form the craniofacial structures migrate from the neural crest, a strip of cells between the newly formed neural tube and the skin of the back. Organs are formed and arranged in a particular way in relation to each other. This formation of tissues and organs, and the sculpting of the general shape of a human being is called morphogenesis. The regulation of cell growth and cell division, as well as controlled cell death (apoptosis) are also essential processes in human development. For example, the sculpting of the limbs and formation of the digits involves programmed cell death. Errors in cell proliferation and apoptosis are seen in cancer, which is why many of the molecules that are important in development are also relevant to cancer research. (Gilbert. 2010)

2.4.1 Neuronal development

The development of the central nervous system take place in consecutive stages that include the induction of neuronal tissue and patterning, the establishment of neuroblasts and their migration, the differentiation of neuronal cell types, axonal guidance, the establishment of synaptic connections, the regulation of cell number by apoptosis, and the arrangement of synaptic connections (Sariola, et al. 2003). The detailed molecular basis of vertebrate motor neuron development described below is largely based on experiments on chick embryos (*Gallus gallus*).

The development of the central nervous system starts during the embryonic stage when a group of ectodermal cells are induced by the notochord, an event called primary induction. Induced neuroepithelial cells form a thickened neural plate, which subsequently folds into the neural groove, and finally closes to form the neural tube (Sadler 2005). Distinct neuronal subtypes are generated in characteristic order along the dorsoventral axis of the developing neural tube (Jessell 2000). The

differentiation of neuronal cells in the developing neural tube is tightly regulated by the expression of specific homeodomain proteins. TGF-β proteins, originating from the dorsal ectoderm, influence the dorsal patterning of the neural tube (Lee and Jessell 1999), while Sonic hedgehog (Shh) protein generated by the notochord and the floor plate of the ventral neural tube controls ventral patterning (Briscoe and Ericson 2001). Different types of neurons are generated from distinct progenitor domains in the ventral neural tube. Shh acts as a graded signal inducing these neuronal subtypes at different concentration threshold and regulates the expression profiles of transcription factors Nkx2.2, Olig2, Nkx6.1, Nkx6.2, Dbx1, Dbx2; Irx3, Pax6, and Pax7. Each progenitor domain expresses a specific combination of these transcription factors (Figure 5) (Briscoe and Ericson 2001, Ribes and Briscoe 2009, Dessaud et al. 2010). The expression of target genes in response to graded Shh signalling in the ventral neural tube is mediated by the transcriptional effector proteins Gli1, Gli2, and Gli3 (Jacob and Briscoe 2003, Vokes et al. 2007). As progenitor cells in the progenitor domains proliferate and exit the cell cycle, they express a combination of LIM homeodomain (LIM-HD) and bHLH transcription factors that define neuronal cell type identity. The anterior horn motor neurons arise from the pMN progenitor domain (Figure 5). The motor neuron progenitor domain expresses the LIM-HD factors Lhx3/Lhx4 and Isl1 that activate motor neuron specific transcription (Ericson et al. 1992, Sharma et al. 1998, Thaler et al. 2002), as well as the bHLH transcription factors Ngn2 and NeuroM (Ma et al. 1996, Roztocil et al. 1997, Scardigli et al. 2001). The coexpression of these molecules marks a transient period in development during which motor neurons begin to acquire general neuronal properties, become postmitotic, and differentiate. The Hb9 protein actively suppresses interneuron differentiation programs in developing motor neurons thus promoting motor neuron development and axon pathfinding (Arber et al. 1999, Thaler et al. 1999) .

Motor neurons in the spinal cord are organized into longitudinally aligned columns, each composed of neurons that innervate the same target tissue. This spatial organization of motor columns serves as basis for classifying spinal motor neuron subtypes (Bonanomi and Pfaff 2010). In the hindbrain, motor neurons arise from two progenitor domains that are adjacent to each other, but genetically distinct; hypoglossal motor neurons arise from Pax6 progenitor cells and coexpress Isl1, Isl2, and transiently, Lhx3 and Gsh4, whilst vagal motor neurons derive from Nkx2.2 progenitor cells and coexpress Isl1 and Gsh4, but not Isl2 or Lhx3 (Ericson et al. 1997). Outgrowth of nerve cell axons is led by the growth cone located on the tip of the axon. During axonal migration, the microspikes of the growth cone enable motility and provide structural support, and also sense the environment and send back signals to the cell soma. Two families of transcription factors have been implicated in the control of motor axon navigation: the 12 member LIM-HD class of

transcription factors and the 39 member HOX family of HD proteins (Tsuchida et al. 1994, Dasen et al. 2005).

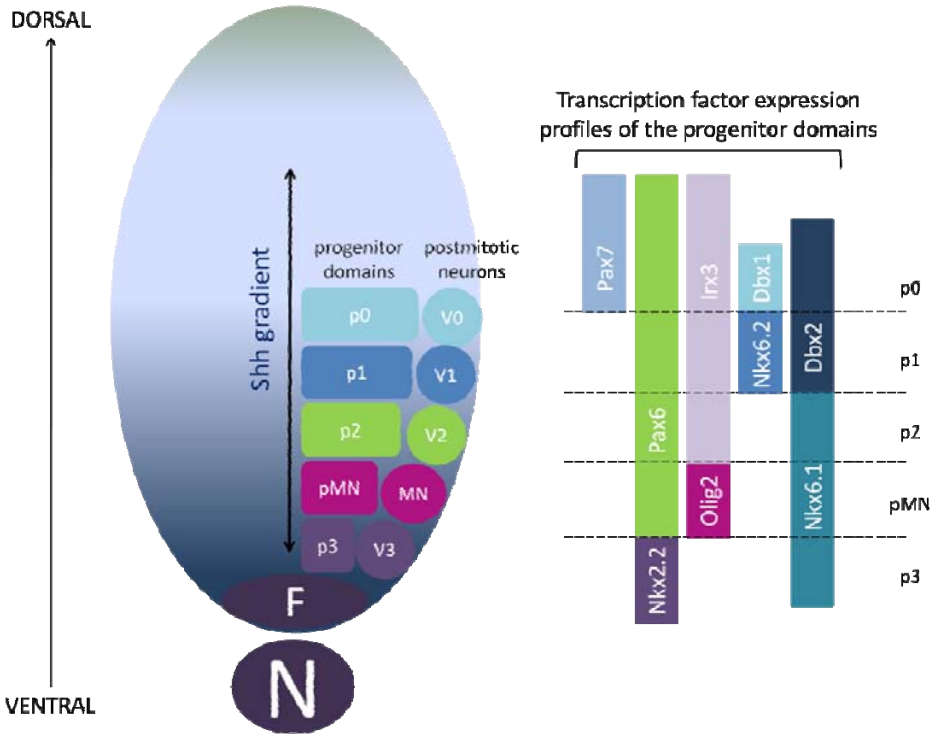


Figure 5. Generation of neuronal diversity in the ventral neural tube in response to graded Shh signalling. N notochord, F floor plate. V0 neurons, V1 neurons, V2 neurons, motor neurons (MN) and V3 neurons are generated at distinct progenitor domains (p3-p0) in the ventral spinal cord. Shh acts as a graded signal inducing these neuronal subtypes at different concentration threshold and regulates the expression profiles of transcription factors Nkx2.2, Olig2, Nkx6.1, Nkx6.2, Dbx1, Dbx2; Irx3, Pax6, and Pax7. Each progenitor domain expresses a unique combination of transcription factors. Modified from (Briscoe and Ericson 2001, Ribes and Briscoe 2009, Dessaud et al. 2010)

The axons of neurons are insulated by a sheath of myelin provided by glial cells that wrap around the axon. In the central nervous system, axon insulation is provided by glial cells called oligodendrocytes, while in the peripheral nervous system, myelination is accomplished by glial cells called Schwann cells. The myelin sheath is essential for normal functioning of neurons. Motoneurons release neuregulin 1 to promote Schwann cell survival and development (Trachtenberg and Thompson 1996, Fischbach and Rosen 1997, Hayworth et al. 2006).

Naturally occurring cell death of neurons during development is regulated by the synthesis and secretion of neurotrophins by neuronal target tissues (Oppenheim 1991). The prototypic neurotrophin is nerve growth factor (NGF), discovered in the 1950s as a soluble peptide (Cohen et al. 1954), promoting the survival of and neurite outgrowth from sympathetic ganglia (Levi-Montalcini 1987). Approximately 50% of motor neurons undergo programmed cell death during the course of development. Since the discovery of NGF, the investigation of this process of physiological motor neuron cell death has resulted in the identification of several neurotrophic factors that influence motor neuron survival, including neurotrophins (Bdnf, Nt-3, Nt4/5), Cntf/Lif family proteins (Cntf, Lif, Ct-1, Clc), Hepatocyte growth factor/scatter factor (Hgf/Sf), Insulin-like growth factors (Igf-1, Igf-2), and Glial-derived neurotrophic factor and related factors (Gdnf, Ntf, Persephin, Artemin) (Sendtner et al. 2000).

The establishment of synaptic connections between muscle tissue and the motor neurons that innervate it requires reciprocal signalling. A neuromuscular junction (NMJ) is a specific type of synapse that forms between motor neurons and skeletal muscle fibers. Multiple neurons compete to form a single neuromuscular junction, but only one neuron that is able to successfully respond to signalling from its target tissue will survive and establish a connection. (Witzemann 2006) In vertebrates, the neuromuscular junction utilizes Acetylcholine (ACh) as a neurotransmitter. Acetylcholine receptors (AChRs) in the muscle fibers are initially present in a broad, poorly defined area in the middle of the muscle fiber, but innervations results in the clustering of AChRs in the synaptic region, and the disappearance of primitive clusters in non-synaptic areas (Wu 2010). Muscle fibers express neurotrophin 3 to modulate the number of Schwann cells in developing NMJs (Hess et al. 2007). Mice lacking Neuregulin 1 or its receptors Erbb2 or Erbb3 lack Schwann cells (Riethmacher and Sonnenberg-Riethmacher 1997, Morris et al. 1999, Woldeyesus et al. 1999, Lin et al. 2000, Wolpowitz et al. 2000), and the synapses formed between their motor neurons and muscle fibers fail to be maintained, suggesting an important role for Schwann cells in the formation and maintenance of neuromuscular junctions. (Wu 2010)

2.4.2 Development of craniofacial structures

The structures of the face and head are mostly derived from the cells of the cranial neural crest. The cranial neural crest cells migrate into the pharyngeal arches and the frontonasal process, and once they have reached their final destination, differentiate to form different craniofacial structures. The migration of these cells is driven by an underlying segmentation process of the hindbrain. The hindbrain is segmented into compartments called rhombomeres, numbered from rhombomere one to rhombomere eight (r1-r8). Neural crest cells from the midbrain and rhombomeres one and two migrate to the first pharyngeal arch and form the jawbones, as well as the incus and malleus bones of the middle ear, and also differentiate into nerve cells that innervate the teeth and the jaw. They also form the frontonasal process, which forms the forehead, the middle of the nose, and the primary palate. Neural crest cells from r4 and r6-r8 contribute to the formation of neck cartilage, the thymus, parathyroid and thyroid glands, and assist in the construction of the aorta and the pulmonary artery (Bockman and Kirby 1984, Serbedzija et al. 1992, Creuzet et al. 2005). The neural crest cells from rhombomeres three and five do not migrate, but undergo apoptosis (Ellies et al. 2002). The skull bones are derived both from the neural crest and the head mesoderm. Studies in transgenic mice suggest that in vertebrates, the bones of the front of the face are generally derived from the neural crest, while the back of the skull originates from a combination of neural crest-derived and mesodermal bones.

The formation of facial features is influenced by various paracrine factors. Wnt signalling induces the protrusion of the frontonasal and maxillary prominences, giving shape to the face (Brugmann et al. 2006). Fibroblast growth factor (FGF) signalling induces neural crest formation (Baker and Bronner-Fraser 1997, Villanueva et al. 2002, Monsoro-Burq et al. 2003, Monsoro-Burq et al. 2005) and promotes the formation of the chondrocyte (i.e. cartilage forming) lineage in the cranial neural crest (Sarkar et al. 2001, Monsoro-Burq et al. 2005). Fibroblast growth factors (FGFs) and their receptors (FGFRs) are broadly expressed in the developing face (Bachler and Neubüser 2001). FGFs are involved in the outgrowth of the facial primordial, craniofacial skeletogenesis, sculpting of the palate, development of teeth and salivary glands, as well as the development of facial muscles (Nie et al. 2006a). The role of FGFs and FGFRs in craniofacial morphogenesis has been widely studied in mouse models. For example, studies on mice indicate that Fgfr1 influences localized signalling in the pharyngeal ectoderm and the development of the second branchial arch (Trokovic et al. 2003, Trokovic et al. 2005).

Another important group of signalling molecules involved in the development of the cranial neural crest and facial primordial are the Bone morphogenic proteins (BMPs).

BMPs work in tandem with FGF and Wnt signalling pathways in the formation of the neural crest and sculpting of the face. BMPs are also involved in craniofacial skeletogenesis and muscle development, and are crucial for the closure of the facial midline. Disturbances in this process result in orofacial clefts such as cleft lip and cleft palate. (Nie et al. 2006b) The critical role of BMPs in facial midline fusion is indicated by studies on mice (Dudas et al. 2004, Liu et al. 2005). Sonic hedgehog (Shh) signalling is also essential for normal craniofacial morphogenesis. Mutations in the human *SHH* gene cause holoprocencephaly, a developmental defect of the forebrain and midface (Roessler et al. 1996).

2.4.3 Development of the epidermis and cutaneous structures

The embryonic ectoderm differentiates to form the nervous system and the epidermis of the skin. Cutaneous structures such as teeth, sweat glands, mammary glands, hair, and nails are also derived from the ectoderm. The skin is the largest organ in the human body. Its outermost layer is the epidermis, a stratified epithelium that consists mostly of proliferating basal and differentiated suprabasal keratinocytes. The epidermis is divided into layers, which are, from the outside inwards:

1. cornified layer (*stratum corneum*)
2. translucent layer (*stratum lucidum*)
3. granular layer (*stratum granulosum*)
4. spinous layer (*stratum spinosum*)
5. basal layer (*stratum basale*)

Normally, the stratified epithelium is maintained by continuous cell proliferation within the basal layer, from where cells are pushed outwards. Once the cells reach the *stratum corneum*, they lose their nuclei, fuse to form squamous sheets, and are eventually shed. This process is called the terminal differentiation program, and it is defined by growth arrest, expression of structural proteins, such as loricrin and filaggrin, and cell death through enucleation (Fuchs and Byrne 1994). In normal skin, the rate of proliferation is equal to the rate of cell loss. The entire epidermis is replaced by new cell growth in approximately 48 days (Iizuka 1994).

The epidermis develops from the surface ectoderm, which initially consists of a multipotent single-layer epithelium. Upon receiving the appropriate developmental cues, these epithelial cells begin to stratify, proliferate, and finally undergo terminal differentiation to produce the morphologically distinct layers of the epidermis (Mack et al. 2005). The epidermal developmental program that is first seen in the fetus is maintained also in the epidermis of the adult organism, where stem cells of the basal layer continuously produce new skin cells.

The transcription factor p63 is a leading candidate for the molecular switch required to initiate stratification of the embryonic single-layered epithelium. It is highly expressed in the epidermal basal layer, and studies in knockout mice suggest that it may also be required to sustain the basal stem cell population (Yang et al. 1998). Notch signalling is implicated in the basal cells' withdrawal from the cell cycle and commitment to terminal differentiation. Conditional elimination of Notch1 from the basal layer of newborn epidermis results in epidermal hyperproliferation, indicating a failure of cells to undergo growth arrest (Rangarajan et al. 2001). In mice, Notch1 is expressed as early as E15.5 in the developing epidermis (Okuyama et al. 2004), indicating that Notch signaling may be important for the initial growth arrest signals that allow the cells to enter a differentiation program. NF-κB transcription factors play an important role in growth arrest and morphogenesis in the developing and renewing epidermis (Mack et al. 2005). CHUK (IKKα), a component of the NF-κB pathway, acts in the nucleus to induce keratinocyte differentiation (Sil et al. 2004), and also functions in epidermal development independently of NF-κB (Hu et al. 2001).

Various Bone morphogenic protein (BMP) ligands and receptors are expressed in different compartments of the developing epidermis. BMP-6 transcript is expressed in the suprabasal layers of the epidermis of mouse embryos starting from E15.5, BMP-7 is seen in the basal layer during the later stages of embryonic development, BMPR-IA is expressed in the basal layer of murine epidermis at E16.5, and BMPR-IB expression is restricted to the suprabasal keratinocytes (Wall et al. 1993, Takahashi 1996, Botchkarev et al. 1999).

HOX genes (*HOXA4, HOXA5, HOXA7*, *HOXB4*, and *HOXC4*) are expressed in human fetal epidermis beginning as early as 10 weeks of gestational age (Stelnicki et al. 1998), suggesting a function early in epidermal development as transcriptional activators of cellular proliferation (Mack et al. 2005).

Cutaneous structures such as hair, sweat glands, mammary glands, and teeth also develop from the embryonic ectoderm. EDA, EDAR, and EDARADD control the development of cutaneous structures via NF-κB signalling (Kere et al. 1996, Ferguson et al. 1997, Srivastava et al. 1997, Headon and Overbeek 1999, Monreal et al. 1999, Headon et al. 2001).

2.4.4 Errors in development

A famous quote by Professor Veronica Van Heyningen states: “The amazing thing about development is not that it sometimes goes wrong, but that it ever succeeds”. It is estimated that more pregnancies are miscarried than are actually carried to term (Rai and Regan 2006). The number is difficult to estimate since often the miscarriage occurs even before the woman is aware of her pregnancy. Most miscarriages are sporadic and non-recurrent, and are often caused by chromosomal abnormalities of the fetus (Dhont 2003). The most common chromosomal abnormalities seen in miscarriages are trisomy of the chromosome 16, followed by trisomy 21 and trisomy 22 (Stephenson and Kutteh 2007). Other commonly seen chromosomal abnormalities associated with miscarriage include polyploidy, monosomy X, and unbalanced translocation (Kalousek et al. 1993, Stephenson et al. 2002). Trisomy 21, found in Down’s syndrome, and monosomy X, found in Turner syndrome, as well as unbalanced translocations, are not invariably fatal, though. Chromosomal abnormalities may arise *de novo*, or they can be due to parental germline mosaicism, or a balanced chromosomal translocation in the parental genome that results in the formation of gametes with an abnormal chromosomal content.

Approximately two percent of human infants are born with an observable anatomical abnormality, a birth defect. Birth defects can be caused by genetic events, such as mutations or an abnormal amount of chromosomes, or environmental factors (teratogens) such as drugs and chemicals (e.g. alcohol, heroin, retinoic acid, antibiotes, thalidomide, and warfarin), ionizing radiation, infectious micro-organisms (e.g. Cytomegalovirus, Herpes simples, Parvovirus, Rubella, and toxoplasmosis), or metabolic conditions in the mother (e.g. autoimmune disease, Diabetes, malnutrition, and Phenylketonuria). The developing fetus is most susceptible to teratogens between three to eight weeks of gestation, a time when organs are forming. However, the nervous system remains susceptible to damage throughout the pregnancy. (Gilbert. 2010)

The formation of the human neural tube begins early during the third week of gestation. The nervous system continues to grow and mature throughout embryonic and fetal development, and connections between neurons integrate the parts of the body and the brain. Failures in this process result in neural tube birth defects such as anencephaly (anterior part of the neural tube fails to close) and spina bifida (posterior part of the neural tube fails to close). Folic acid, or vitamin B9, is known to be an important molecule in the closure of the neural tube, and obtaining sufficient amounts of this vitamin is essential for pregnant women. (Gilbert. 2010)

2.5 Animal models

Various different animal models are used in biological and biomedical research. The budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*) are employed in the study of basic molecular and cellular mechanisms of the eukaryotic cell. The roundworm (*Caenorhabditis elegans*) has been particularly useful in studying cell fate and apoptosis during development. The Africal clawed frog (*Xenopus laevis*) has been an important model in developmental biology because of its large and easily manipulable embryo, but it has a tetraploid genome, which makes it a less desirable candidate for genetic studies. A related frog species, the *Xenopus tropicalis*, which has a simpler genome, is now widely used. The chicken (*Gallus gallus)* has also been widely used in developmental research, particularly the study of limb formation.

The mouse (*Mus musculus*) is the most popular mammalian model used in biological and biomedical research. Gene targeting in mouse embryonic stem cells is a technique of engineering specifically tailored mutant mice that enables the generation of gene-specific knockouts or knock-ins, as well as tissue-specific expression of mutant genes (Capecchi 2005). The Mouse Genome Database is an online resource of mouse genomics, gene expression, and mutant mouse phenotypes (Bult et al. 2008).

The zebrafish (*Danio rerio*) has many qualities that make it a practical and widely used model organism in the study of development and disease. Zebrafish embryos develop externally and can be made optically transparent by inhibiting pigment formation, which enables direct monitoring of their development *in vivo*, and the use of fluorescent reporters and indicators to visualize morphology and physiology of specific cell groups of interest (Sager et al. 2010). The fish breed regularly, produce large amounts of offspring, and can be housed in large numbers enabling large-scale genetic screens (Brockerhoff et al. 1995, Driever et al. 1996, Amsterdam et al. 1999, Amsterdam et al. 2004) and chemical screens (Zon and Peterson 2005). The zebrafish genome has been duplicated during the course of evolution, which makes genetic studies challenging since some, but not all zebrafish genes are present in multiple copies (Amores et al. 1998). Genetic manipulation techniques, such as the use of morpholino antisense oligonucleotides, can be employed to investigate the function of a particular gene during zebrafish development (Eisen and Smith 2008). The zebrafish genomic sequence and other resources are available online (www.zfin.org) (Sprague et al. 2006).

2.5.1 The Zebrafish as a model of neurodegeneration

The zebrafish central nervous system (CNS) is considerably smaller than the human CNS, and has some structural differences as well. However, many areas of the zebrafish CNS that are relevant in terms of disease are structurally similar to those in humans. For example, both the zebrafish and human cerebellum have molecular, Purkinje cell and granule cell layers. The cell types present in the each of these layers are alike in zebrafish and humans; they show similar inputs and synaptic connections, and express similar genes and specialized markers (Bae et al. 2009). One difference is that the cell bodies of output projection neurons in the zebrafish cerebellum (eurydendroid cells) are located in the cortex rather than the deep nuclei found in humans. However, other regions of the zebrafish CNS, such as the medulla, hypothalamus, optic tracts and tectum, olfactory system, spinal cord, and cranial nerves show easily recognizable structural homology to their human counterparts. (Sager et al. 2010)

In order to investigate whether the zebrafish could be used as a model organism for studying the pathogenesis of ALS, a study used mRNA microinjection to cause transient over-expression of mutant *SOD1* in developing zebrafish embryos (Lemmens et al. 2007). The microinjected animals showed normal morphology and normal development of Mauthner neurons, Rohan-Beard sensory neurons and lateral line sensory neurons. However, the motor neurons in the microinjected animals displayed abnormal branching and shortened axon length (Lemmens et al. 2007). Knocking down the survival motor neuron (*Smn*) gene, which is mutated in human patients with Spinal Muscular Atrophy (SMA), causes defects in motor axon outgrowth and pathfinding in zebrafish (McWhorter et al. 2003). Thus the zebrafish can be considered a good model organism for the study of human motor neuron disease, where often the ubiquitous expression of a mutant protein results in a motor neuron-specific neuropathology.

3 Aims of the study

The aims of this study were the following:

1. To identify the molecular defect behind LCCS1
2. To identify the molecular defect behind LAAHD
3. To describe in detail a familial case of fetal encasement malformation
4. To investigate the molecular background of the fetal encasement malformation

4 Subjects, materials and methods

4.1 Subjects and samples

Samples for the study were available from 29 LCCS1 families, with 52 affected fetuses and 73 healthy individuals (including parents and healthy siblings). There were ten LAAHD families with a total of thirteen affected individuals. DNA was extracted from blood, frozen and paraffin-embedded tissue, cultured cells, and chorionic villus samples. RNA was extracted from frozen tissue and cultured cells. A fibroblast cell line was available from two LCCS1 fetuses and from one fetus of North American origin, whose phenotype was similar to LCCS1. In addition, we analyzed samples from ten foreign families with suspected cases of LCCS1 or LAAHD.

For the fetal encasement malformation study, samples were available from one family with two affected fetuses and their parents. DNA was extracted from blood and cultured cells, and RNA from cultured cells. Fibroblast cell lines and tissue samples were available from both affected fetuses.

In addition, DNA was available from 200 Finnish control samples.

Fetal DNA, tissue samples and fibroblast cell lines were available from healthy fetuses aborted for non-medical reasons.

4.1.1 Ethical aspects

Informed written consent was obtained from the parents of the families participating in the study. The studies were approved by the Ethical Committees of the Joint Authority for the Hospital District of Helsinki and Uusimaa.

4.2 Methods

4.2.1 Methods used in original publications

The methods used in this study are listed in Table 5 and described in detail in the original publications.

Table 5. The original publications in which the methods are used are indicated with Roman numerals.

Method	Publication
Bioinformatic analyses	I, II
Cell culture	I, II
Cell transfections	I
Direct sequencing	I, II
DNA extraction	I, II
Genealogy	II
Genotyping	I
Haplotype analyses	I
Immunofluorescence microscopy	I
Immunofluorescence staining	I
Microarray analysis	II
Polymerase chain reaction (PCR)	I, II
Protein immunoprecipitation	II
Reverse transcription polymerase chain reaction (RT-PCR)	I, II
RNA extraction	I, II
RNA *in situ* hybridization	I
Western Blot	II

4.2.2 Materials and methods used in unpublished studies

Mutant gle1$^{-/-}$ zebrafish embryos

Two strains of *gle1* $^{+/-}$ zebrafish were available for studies at the Wellcome Trust Sanger Institute, Cambridge, UK. One of these strains containing a transgenic insertion, which disrupts the *gle1* gene, was previously identified in a scan for developmental genes in zebrafish (Amsterdam et al. 2004). The other strain, which carries a truncating point mutation in *gle1*, was identified in a genetic screen at the Vertebrate development and genetics research group at the Wellcome Trust Sanger Institute (Dr. Derek Stemple and Dr. Richard White, personal communication). Wild type AB zebrafish were available as controls. Fish strains were maintained in the Wellcome Trust Sanger Research Support Facility (RSF) in accordance with UK laws and regulations concerning experiments with animals, and in compliance with the ethical guidelines issued by the Wellcome Trust Sanger Institute.

Acridine orange (AO) staining of gle1$^{-/-}$ zebrafish embryos

Adult zebrafish were set up to mate in breeding pairs, and fertilized embryos were collected from the bottom of the breeding tanks. Embryos were reared in egg water (60 μg/ml sea salt, 0.0002% methylene blue) containing 0.2 mM 1-phenyl 2-thiourea (PTU). PTU was used to inhibit pigment formation in embryos in order to obtain better images. Mutant *gle1*$^{-/-}$ embryos could be distinguished from siblings by viewing them under the microscope due to the obvious phenotype of markedly reduced movement compared to the wild type siblings, a small head, and small eyes. Identification was verified by genotyping mutant and wild type embryos, and the results were consistent with the visual identification. Images of embryos were taken at two days post fertilization (2dpf). Prior to imaging, the egg water containing the embryos was supplemented with Acridine orange (AO) to a final concentration of 5μg/ml, and allowed to stand for 30 minutes. The egg water was then changed a few times to wash off excess AO, and embryos were mounted in methyl cellulose for live imaging on the Leica TCS SP5 II broadband confocal microcope.

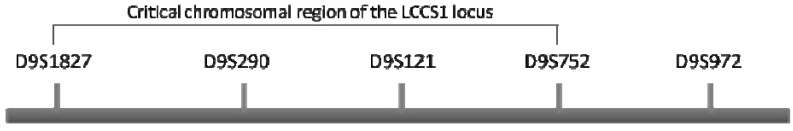
Whole mount Poly-A in situ hybridization of zebrafish embryos

Localization of polyadenylated RNA was assayed by *in situ* hybridization. A digoxygenin-tailed poly(dT)$_{50}$ oligonucleotide probe was used to detect polyadenylated messenger RNA (Forrester et al. 1992, De Bruyn Kops and Guthrie 2001), and digoxygenin-labelled RNA probes synthezised in the antisense- and sense direction from a plasmid containing a fragment of the *dbx1a* gene (Gribble et al. 2009), whose expression pattern in the zebrafish embryo is distinct and well studied, were used as positive and negative controls for the hybridization method. Whole mount *in situ* hybridization was carried out as described previously (Thisse and Thisse 2008). After probe hybridization, staining was done with anti-digoxygenin conjugated with horseradish peroxidase (anti-DIG-POD) and Tyramide Signal Amplification TSA Cy3 substrate (Perkin Elmer). Embryos were also stained with DAPI to visualize the nuclei within the individual cells. Images were taken with the Leica TCS SP5 II broadband confocal microscope.

5 Results and discussion

5.1 Identification of the LCCS1 disease gene (I)

Prior to this thesis work, the LCCS1 locus had been mapped to chromosome 9q34 by linkage (Mäkelä-Bengs et al. 1998). The critical chromosomal region for the disease gene was restricted between markers D9S1827 and D9S752 based on recombinations in a shared ancestral haplotype observed in LCCS1 families (Figure 6). Some candidate genes had also been sequenced (Table 6) (Pakkasjärvi 2005b). This sequencing work had been done simultaneously with the progress of the Human Genome Project at a time during which a conclusive genetic map of the region was not yet available. Although the HGP had officially been completed, the genetic map of the LCCS1 region in chromosome 9q34 was still updated on a regular basis even during the course of this thesis work, with discovery of new transcripts in the region, and changes is the number of coding exons within existing transcripts, which made it essential, but also challenging to keep track of what had already been sequenced. Gene nomenclature also changed during the course of the work (Table 6).



Analyzed markers on chromosome 9q34					no. of chr
200	164	143	460	150	23
198	164	143	460	150	6
196	164	143	460	150	1
202	164	143	460	150	1
202	164	143	460	140	1
198	164	143	460	146	1
200	164	143	474	140	1
200	164	143	460	146	2
200	164	143	460	142	2

Figure 6. Recombinations in the shared ancestral haplotype restrict the critical chromosomal region of the LCCS1 locus.

Table 6. Candidate genes sequenced prior to this study. The left-hand column lists the gene names that were valid in the year 2004, in which this thesis work was started. The right-hand column lists the same genes by their current official names as stated in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov)

2004		**2010**	
Gene	**Full name**	**Gene**	**Full name**
LCN2	lipocalin 2	***LCN2***	lipocalin 2
STXBP1	syntaxin binding protein 1	***STXBP1***	syntaxin binding protein 1
ZNF-X	zinc finger X transcription factor	***ZBTB43***	zinc finger and BTB domain containing 43
DNM-1	dynamin 1	***DNM1***	dynamin 1
KIAA1069	novel gene	**–**	not found in NCBI database
CCBL	cysteine conjugate-beta lyase	***CCBL1***	cysteine conjugate-beta lyase, cytoplasmic
SPTAN	brain spectrin alpha / fodrin	***SPTAN1***	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
ZYG	ZYG-11 homolog B	***ZER1***	zer-1 homolog (C. elegans)
ENDOG	endonuclease G	***ENDOG***	endonuclease G
VSN	novel gene	**–**	not found in NCBI database
SH3GLB2	endophilin B2	***SH3GLB2***	SH3-domain GRB2-like endophilin B2
CEECAM1	cerebral endothelial cell adhesion molecule 1	***CERCAM***	cerebral endothelial cell adhesion molecule
DOLPP1	dolichyl pyrophosphate phosphatase 1	***DOLPP1***	dolichyl pyrophosphate phosphatase 1
CRAT	carnitine acetyltransferase	***CRAT***	carnitine O-acetyltransferase
LRRC8	leucine-rich repeat-containing protein 8	***LRRC8A***	leucine rich repeat containing 8 family, member A
PHYHD1	phytaonyl-CoA dioxygenase domain containing 1	***PHYHD1***	phytanoyl-CoA dioxygenase domain containing 1
IER5L	immediate early response gene 5L	***IER5L***	immediate early response 5-like
PPP2R4	phosphotyrosyl phosphatase activator 2A	***PPP2R4***	protein phosphatase 2A activator, regulatory subunit 4

The size of the LCCS1 region in chromosome 9q34 between markers D9S1827 and D9S752 is 1 Mb, and it contains 30 protein-coding genes (UCSC Human Genome Browser, February 2009 Assembly) (genome.ucsc.edu) (Figure 7 and Table 7). Searching for a causative mutation by sequencing regional candidate genes of three affected LCCS1 individuals and two controls revealed that the region was very homogeneous. While searching for the causative mutation, we also searched for polymorphisms that would segregate with the LCCS1 disease haplotype, and could potentially be used to further restrict the critical chromosomal region, but discovered only SNPs that were present in both patients and controls, and thus most likely represent ancient polymorphisms that were introduced to the Finnish population before the appearance of the LCCS1 disease mutation.

Eventually, we found one previously unidentified SNP in the 3'UTR (untranslated region) of the *SLC27A4* gene, which seemed to segregate with the disease. All affected LCCS1 individuals in 28 families were homozygous, and their parents heterozygous for the G allele of this SNP. In the one remaining LCCS1 family, the fetus was heterozygous for the G allele, and the disease associated ancestral haplotype was observed in only one parent, who was also heterozygous for the G allele. Of 200 healthy controls, 1% were heterozygous for the disease associated G allele, but none were homozygous. A theoretical estimate of the carrier frequency of the LCCS1 disease mutation, which can be calculated using the Hardy-Weinberg equation and the observed incidence of LCCS1 in the Finnish population (1:25,250), is 1,3%, which is relatively close to the observed 1% in our 200 controls. The location of this SNP in the 3'UTR of the gene did not make it the most likely candidate for a pathogenic mutation, even though the polymorphism segregated with the LCCS1 phenotype. However, a mutation in the non-coding region of a gene might still affect the expression of the transcript, for example by creating a micro-RNA binding site, and therefore, at this stage, the SNP could not be completely ruled out as a possible disease-causing mutation.

Because of the phenotypic similarity between LCCS1 and LAAHD, we next investigated the LCCS1 disease region and the newly identified *SLC27A4* polymorphism in LAAHD families. Comprehensive genotyping of all the LAAHD families was not possible due to the poor quality of some of the DNA samples, which were mostly extracted from paraffin blocks. To investigate the *SLC27A4* polymorphism in cases with LAAHD we designed a special PCR with a very short amplicon, which enabled the analysis of this SNP also in low quality LAAHD DNA samples extracted from paraffin-embedded tissue. Interestingly, almost all of the LAAHD fetuses were also heterozygous for this SNP, except for a pair of identical twins, whose phenotype was somewhat intermediate between LCCS1 and LAAHD,

and who were heterozygous for the shared ancestral LCCS1 haplotype, but homozygous for the wild type A allele of the SNP. This suggested that the polymorphism was most likely not pathogenic, but rather in almost complete LD with the true disease-causing mutation. Given the low incidence of this SNP in the general population and its frequent occurrence in LAAHD families, we concluded that LCCS1 and LAAHD might be allelic, and thus the recombination observed in one LAAHD family enabled us to further restrict the critical chromosomal region to 800,000 bp, reducing the amount of regional candidate genes to 24 (Figure 7).

We screened all of the regional candidate genes for possible disease causing mutations by direct sequencing and found potential mutations in only one of them, *GLE1*. In 28 out of 29 LCCS1 families, affected fetuses were homozygous and parents heterozygous for a point mutation in intron 3 of the *GLE1* gene, ten base pairs upstream of exon 4 (c.433-10A>G). In one LCCS1 family, where the shared ancestral haplotype was inherited only from one parent, the fetus was a compound heterozygote for the c.433-10A>G mutation and a missense mutation in exon 12 (c.1706G>A).

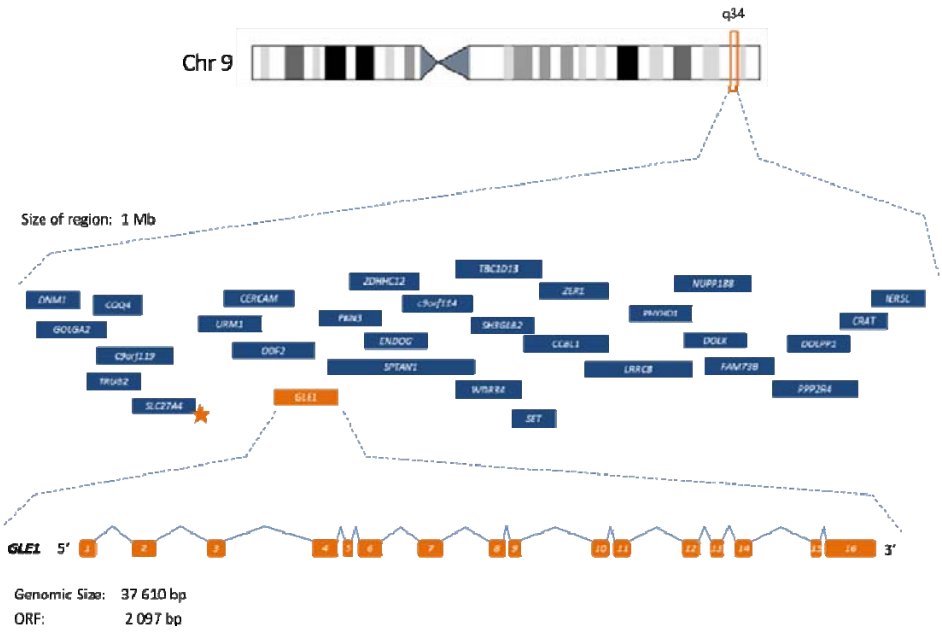


Figure 7. The critical chromosomal region for the LCCS1 locus. A polymorphism in the *SLC27A4* gene restricted the critical chromosomal region to 800,000 bp, and mutations in LCCS1 patients were found in the *GLE1* gene.

Table 7. List of protein coding genes located in the LCCS1 locus between markers D921827 and D9S752 according to UCSC Human Genome Browser February 2009 assembly. Genes sequenced from genomic DNA prior to this study are indicated in grey. These genes were resequenced from cDNA during this study.

Gene	Full name
DNM1	dynamin 1
GOLGA2	golgin A2
c9orf119	chromosome 9 open reading frame 119
TRUB2	TruB pseudouridine (psi) synthase homolog 2 (E. coli)
COQ4	coenzyme Q4 homolog (S. cerevisiae)
SLC27A4	solute carrier family 27 (fatty acid transporter), member 4
URM1	ubiquitin related modifier 1
CERCAM	cerebral endothelial cell adhesion molecule
ODF2	outer dense fiber of sperm tails 2
GLE1	GLE1 RNA export mediator homolog (yeast)
SPTAN1	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
WDR34	WD repeat domain 34
SET	SET nuclear oncogene
PKN3	protein kinase N3
ZDHHC12	zinc finger, DHHC-type containing 12
ZER1	zer-1 homolog (C. elegans)
TBC1D13	TBC1 domain family, member 13
ENDOG	endonuclease G
c9orf114	chromosome 9 open reading frame 114
CCBL1	cysteine conjugate-beta lyase, cytoplasmic
LRRC8	leucine rich repeat containing 8 family, member A
PHYHD1	phytanoyl-CoA dioxygenase domain containing 1
DOLK	dolichol kinase
NUP188	nucleoporin 188kDa
SH3GLB2	SH3-domain GRB2-like endophilin B2
FAM73B	family with sequence similarity 73, member B
DOLPP1	dolichyl pyrophosphate phosphatase 1
CRAT	carnitine O-acetyltransferase
PPP2R4	protein phosphatase 2A activator, regulatory subunit 4
IER5L	immediate early response 5-like

5.1.1 Screening LAAHD families for mutations in *GLE1*

We sequenced the *GLE1* gene from twelve LAAHD fetuses in nine families. Six of these fetuses were compound heterozygous for the LCCS1 Fin_{Major} mutation (c.433-10A>G) and a missense mutation in exon 13 (c.1849G>A), and the remaining six were compound heterozygous for the LCCS1 FinMajor mutation (c.433-10A>G) and a missense mutation in exon 16 (c.2051T>C). We also analyzed the DNA of one patient who had congenital arthrogryposis, but survived for two months after birth. An initial diagnose of spinal muscular atrophy (SMA type I or type 0) was made in this case, but no SMN gene deletions were found. The autopsy showed neurogenic muscle atrophy and loss of anterior horn cells of the spinal cord. This case was also compound heterozygous for the LCCS1 FinMajor mutation (c.433-10A>G) and the mutation in exon 16 (c.2051T>C), and thus the diagnosis was amended as LAAHD (albeit with unusually prolonged survival). Thus the mutation analysis of the LAAHD samples confirmed that LCCS1 and LAAHD are allelic disorders, and that the compound heterozygous mutations in *GLE1* seem to result in a milder phenotype than the homozygous LCCS1 FinMajor mutation. So far, we have not observed the amino acid substitutions of exon 12, exon 13, or exon 16 in a homozygous state in any individuals. It would be extremely interesting to see what kind of phenotype such a mutation would cause.

After the publication of Original publication I, LAAHD was assigned with a MIM number (MIM 611890).

5.1.2 The effect of the mutations on the GLE1 protein

Sequencing of cDNA of the LCCS1 fetuses revealed that the intronic (c.433-10A>G) mutation creates an illegitimate splice acceptor site and results in the insertion of nine extra nucleotides into *GLE1* mRNA. Since exon three ends in a full codon, the aberrant splicing does not disrupt the reading frame, but results in the insertion three extra amino acids (proline, phenylalanine, and glutamine) into the GLE1 protein. Figure 2 in Original Publication I illustrates the structure of the *GLE1* gene and the GLE1 protein in detail. The GLE1 protein contains multiple domains, including the putative coiled coil domain. Coiled coils are secondary, alpha-helical protein structures that contain a characteristic heptad repeat motif $(abcdefg)_n$, and can be reliably predicted from sequence (McDonnell et al. 2006). We used the Paircoil2 algorithm (groups.csail.mit.edu/cb/paircoil2/) to compare predicted coiled coil structures of the mutant and wild type GLE1 polypeptides, and observed that the mutant protein is less likely to form a coiled coil structure than the wild type protein (Original Publication I, Supplementary Table 4). One residue that could have a significant impact on secondary protein structure of the mutant protein is proline, whose cyclic structure represents exceptional conformational rigidity compared to

other amino acids. When proline occurs in the middle of alpha helixes, it usually disrupts the helical structure.

For evaluating the missense mutations in exon 12, exon 13, and exon 16, we used the PolyPhen bioinformatics tool (genetics.bwh.harvard.edu/pph/) (Ramensky et al. 2002), which predicts the possible effects of the amino acid substitution on the protein by the basis of protein sequence annotation, multiple sequence alignments, and structural information. PolyPhen classifies mutations as benign (most likely lacking any phenotypic effect), possibly damaging (supposed to affect protein function or structure), or probably damaging (with high confidence supposed to affect protein function or structure). Table 8 lists the mutations observed in LCCS1 and LAAHD patients and their predicted effects on the GLE1 protein.

Table 8. Mutations found in LCCS1 and LAAHD patients and their predicted effects on the GLE1 protein. A novel mutation described in section 5.1.3. is also included in the table.

Mutation	Predicted effect on polypeptide	Exon	Intron	Predicted consequences
c.433-10A>G	p.T144_E145insPFQ		3	Destroys coiled coil domain (Paircoil2)
c.1706G>A	p.R569H	12		Probably damaging (PolyPhen)
c.1849G>A	p.V617M	13		Benign (PolyPhen)
c.2051T>C	p.I684T	16		Possibly damaging (PolyPhen)
c.581+1_581+2delGT			4	missplicing

5.1.3 Screening additional patients for mutations in *GLE1* (unpublished data)

We screened ten foreign families with suspected LCCS1 or LAAHD diagnosis from various different ethnic backgrounds for mutations in *GLE1*, but found no pathogenic mutations in them. In addition, we sequenced a fetus of Finnish origin with suspected LCCS1, whose sample we received after the Original publication 1, and observed that the sample was a compound heterozygote for the mutation in *GLE1* exon 13 and a novel mutation that abolishes an existing splice donor site in the exon 4/intron 4 junction of *GLE1*. The novel mutation is a deletion of two nucleotides (c.581+1_581+2delGT). Analysis of patient cDNA by agarose gel electrophoresis confirmed that this mutation results in aberrant splicing of *GLE1* mRNA.

5.2 *GLE1* function

The *GLE1* gene has two splice variants that encode two different forms of GLE1 protein, GLE1A and GLE1B. GLE1A is encoded by the first 14 exons of the *GLE1* gene, whereas GLE1B is encoded by all 16 exons. The two proteins are identical except for their C-terminal regions (Kendirgi et al. 2003). Since some patients with LAAHD are compound heterozygotes for the LCCS1 FinMajor mutation and an amino acid substitution in exon 16 of *GLE1*, while others are compound heterozygous for the LCCS1 FinMajor mutation and an amino acid substitution in exon 13, and there are no obvious phenotypic differences between these two types of LAAHD patients, it is reasonable to assume that the full length isoform of GLE1 (GLE1B) is more relevant in terms of the disease mechanism.

Both GLE1 isoforms localize diffusely in the cytoplasm, but GLE1B is also localized in the nuclear envelope and the nuclear pore complex (Rayala et al. 2004). GLE1B forms a heterotrimeric complex with nucleoporins NUP155 and NUPL2 (alias CG1) *in vitro* and is required for the export of heat shock protein 70 (HSP70) mRNA from the nucleus to the cytoplasm in budding yeast as well as in human cells (Kendirgi et al. 2003, Kendirgi et al. 2005). The highly conserved C-terminal NUPL2-binding domain of GLE1B is required for the localization of GLE1B to the nuclear pore complex (Rayala et al. 2004). A small organic molecule, inositol hexakisphosphate (IP_6) binds directly to the yeast homologue of GLE1, and together they stimulate the ATPase activity of DEAD-box protein DBP5 for mRNA export from the nucleus to the cytoplasm. Importantly, although GLE1 alone can enhance the ATPase activity of DBP5, the ATPase activity is maximally stimulated by the presence of both GLE1 and IP6 (Tran et al. 2007). The C-terminal domain of GLE1 is also required for interactions with DBP5 (Alcazar-Roman et al. 2006, Weirich et al. 2006).

Studies in the budding yeast have shown that in addition to mRNA export, Gle1 also functions in translation initiation and translation termination. Studies suggest that Gle1 operates together with inositol hexakisphosphate and Dbp5 in translation termination, whereas IP_6 and Dbp5 are not required for the translation initiation function of Gle1. Gle1 also has a conserved physical association with the eukaryotic translation initiation factor eIF3 and the translation termination factor Sup45. (Bolger et al. 2008)

5.2.1 Overexpression of mutant and wild type GLE1 in HeLa-cells

We cloned the wild type and LCCS1 Fin_{Major} mutant *GLE1* cDNA into expression vectors containing a gene for Green fluorescent protein (GFP) and overexpressed the GLE1-GFP constructs in HeLa cells. There was no difference between the intracellular localization of the mutant and wild type GFP fusion proteins (Original publication I, Supplementary figure 3). The localization for both proteins was similar to that observed in previous studies of wild type GLE1 overexpression in HeLa cells (Kendirgi et al. 2003). The overexpression of a fusion protein, however, does not necessarily reflect the endogenous cellular localization of the protein of interest. At the time of these overexpression studies, there were no GLE1 antibodies available. Now that GLE1 antibodies have become commercially available, it would be interesting to stain LCCS1 patient cells and control cells for endogenous GLE1 and see, whether there is a difference in the expression or localization of endogenous GLE1 protein between LCCS1 patients and controls.

5.2.2 *In situ* hybridization

We performed RNA *in situ* hybridization to investigate the expression of wild type *Gle1* during mouse embryonic development. RNA *in situ* hybridization on sagittal sections of E11 and E13 (E = embryonic day) mouse embryos showed low, ubiquitous expression of *Gle1*. In addition to this, expression was also detected in somites, from which skeletal muscle and bone tissue differentiate, as well as in lung epithelial cells, intestinal wall, esophagus, and brain, specifically the developing choroid plexus. Marked expression was seen in the ventral cell population of the neural tube in the E11 embryo. (Original publication I, Supplementary figure 2)

As mentioned in section 2.4.1, the differentiation of neuronal cells in the developing neural tube is tightly regulated by the expression of specific homeodomain proteins. There is a gradient of Sonic hedgehog (Shh) protein that regulates the expression of transcription factors, such as Nkx2.2 and Pax6. Shh concentration is highest in the ventral cell population of the neural tube that is closest to the notochord, and becomes more diluted once distance from the notochord increases. (Briscoe and Ericson 2001)

In our previous studies of global transcript profiles of the spinal cords of LCCS1 fetuses we did not observe any significant change in the steady state transcript level of *GLE1,* but we detected marginal down-regulation of *NKX2.2* and up-regulation of *PAX6* (Pakkasjärvi et al. 2007).

5.2.3 LCCS1, LCCS, and LCCS3 disease genes are linked by a common pathway

Inositol hexakisphosphate (IP_6) is a member of the inositol polyphosphates, a group of small molecules that participate in various functions in the cell (see section 2.3.6.). The LCCS2 and LCCS3 disease genes belong to the biosynthetic pathway that is responsible for the production of inositol hexakisphosphate (Figure 8). Studies in the budding yeast have shown that yeast strains lacking enzymes in this pathway have reduced interactions between Gle1 and Dbp5 (Weirich et al. 2006). Thus it is possible that there is a common disease mechanism in these three lethal motoneuron diseases, and that this disease mechanism is related to the functions of GLE1.

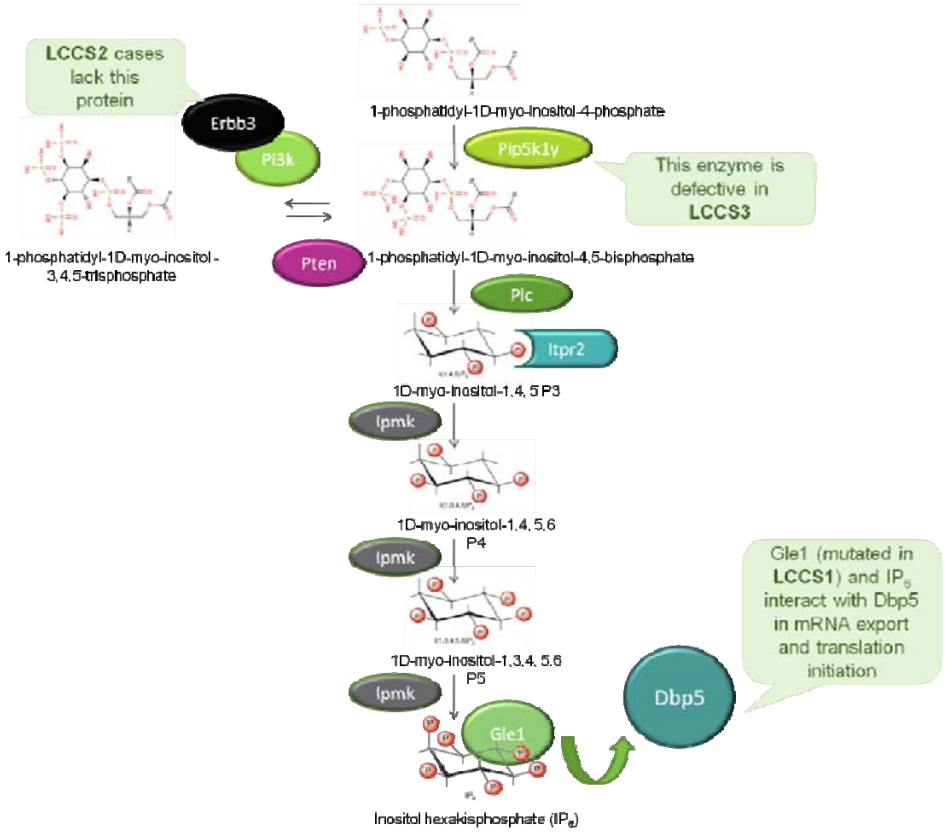


Figure 8. Studies in the budding yeast (*Saccaromyces cerevisiae*) suggest that the causative genes for LCCS1, LCCS2, and LCCS3 are all members of the same pathway.

5.2.4 Posttranscriptional RNA processing

The eukaryotic messenger RNA (mRNA) is transcribed in the nucleus, processed by the splicing machinery, and actively transported to the cytoplasm, where it is translated into protein, and eventually degraded by exonucleases. Throughout this process, mRNAs are accompanied by a host of associated factors, some of which are stably bound while others are subject to dynamic exchange (Moore 2005). The budding yeast is estimated to express almost 600 proteins that have the capacity to bind RNA (Costanzo et al. 2001). In humans this number is thought to be considerably larger, since just a single type of RNA binding domain, the RNA recognition motif (RRM), is present in nearly 500 human genes (Maris et al. 2005), and in addition to this, there are at least five more types of common RNA binding domains found in human genes (Messias and Sattler 2004, Stefl et al. 2005).

The mRNA, associated proteins, and small noncoding RNAs, make up the messenger ribonucleoprotein particle (mRNP). Some mRNAs travel as individual mRNPs, while others cluster and migrate in larger ribonucleoprotein particle (RNP) structures. In neurons, such particles can contain roughly 30 mRNAs and have diameters up to 1 µm (St. Johnston 2005). Some mRNP components, such as nucleocytoplasmic shuttling hnRNPs (heterogeneous nuclear RNPs), are first assembled in the nucleus, simultaneously with transcription and pre-mRNA processing, and accompany the mature mRNA into the cytoplasm. The mRNP helps to regulate the spatial and temporal expression of mRNA within the cell (Martin and Ephrussi 2009). The individual mRNP components allow mRNAs to interface with the intracellular machineries that regulate their subcellular localization, translation, and decay (Moore 2005). The assembly of mRNPs, their export into the cytoplasm through the NPC, and mRNP localization to specific compartments of the cell thus represent a central step in the regulation of gene expression.

The mRNPs are actively exported from the nucleus to the cytoplasm through the nuclear pore complex. Export of mRNPs through the NPC is coincident with altered protein-protein and protein-RNA interactions in the mRNP complex. The DEAD box protein DBP5, GLE1, and inositol hexakisphosphate (IP_6) are essential mRNA export factors at the nuclear pore required for mRNP remodeling and the nucleosytoplasmic transport of mRNA (Tran et al. 2007). Once they have reached the cytoplasm, individual mRNP particles can either passively diffuse until they are trapped by an anchor protein, or be actively transported into subcellular localizations along the cytoskeleton by myosins, dyneins, or kinesins (St. Johnston 2005). Once the mRNAs enter the cytoplasm, not all are immediately translated into protein. Some mRNAs remain actively repressed until they arrive at a specific location in the cell where protein synthesis can take place.

Eukaryotic translation takes place in four distinct stages: initiation, elongation, termination, and recycling (Kapp and Lorsch 2004). Eukaryotic initiation factors (eIFs) that stimulate ribosome loading are key molecules in translation initiation, which involves the assembly of translation-competent ribosomes on mRNA. One of these initiation factors, the eIF3, also regulates dissociation and recycling of the ribosome after translation termination (Pisarev et al. 2007). The termination of translation and the release of the completed polypeptide, also a critical step in gene expression, is regulated by proteins called eukaryotic release factors (eRFs) (Kapp and Lorsch 2004).

Localized mRNA translation is particularly important to large cells such as neurons. During brain development, local translation of mRNAs in axonal growth cones allows neurons to respond to local environmental cues as the distal axonal processes navigate toward their synaptic partners, and in the mature brain, localized mRNA synthesis enables synaptic plasticity (Martin and Ephrussi 2009).

5.2.5 RNA processing and motor neuron disease

The discovery of defective GLE1 behind the molecular pathogenesis of LCCS1 and LAAHD, as well as other recent advances in motor neuron disease genetics, highlight the importance of RNA processing in the pathogenesis of motor neuron disease.

Spinal Muscular Atrophy (SMA) is caused by mutations in the *SMN1* gene, which encodes the Survival of motor neurons (SMN) protein. SMN is found in both the nucleus and the cytoplasm of the cell. In the nucleus, SMN localizes in specks called “gems” that overlap or are closely associated with Cajal bodies, structures that contain high levels of factors involved in transcription and processing of many types of nuclear RNA (Liu et al. 1997). In addition, SMN is found in granules in the axons of neurons, where it is rapidly transported bi-directionally (Zhang et al. 2003, Zhang et al. 2006). SMN is also enriched at the growth cone of motor neurons (Rossoll et al. 2003). Studies suggest that SMN is involved in diverse aspects of RNA metabolism, including pre-RNA splicing, transcription, and metabolism of ribosomal RNAs. (Sumner 2007)

Spinal Muscular Atrophy with Respiratory Distress (DSMA1, MIM 604320) is another severe early onset motor neuron disease, and is caused by mutations in the *IGHMBP2* gene. The protein encoded by this gene has a helicase domain, and it co-localizes with the RNA-processing machinery in both the cytoplasm and the nucleus (Miao et al. 2000).

The rare childhood or adolescent-onset autosomal dominant form of Amyotrophic Lateral Sclerosis ALS4 (MIM 602433) is caused by mutations in *SETX*, encoding senataxin, a protein containing a C-terminal motif typical for DNA/RNA helicases. The *SETX* gene shares homology with the *IGHMPB2* gene and also with *RENT1*, both encoding proteins with a role in RNA processing. Mutations in the *TARDBP* and *FUS* genes have also been found in ALS patients. The *TARDBP* gene encodes for the TDP43 protein, which contains two RNA recognition motifs, and a C-terminal hnRNP-interacting domain. TDP43 shuttles between the nucleus and the cytoplasm, and has a role in RNA processing as well as the regulation of alternative splicing (Buratti and Baralle 2010). FUS, like TDP43, also binds RNA, shuttles between the nucleus and the cytoplasm, and has multiple functions involving mRNA processing (Buratti and Baralle 2010). These characteristics of TDP43 and FUS are strikingly similar with each other, as well as with the attributes and cellular functions of GLE1. These discoveries have shifted the focus of cellular studies of ALS pathogenesis from the extensively studied superoxide dismutase SOD1 enzyme to RNA processing events, possibly leading to novel therapeutic approaches in treating ALS.

5.2.6 *gle1* $^{-/-}$ zebrafish (unpublished data)

Mating of heterozygous *gle1*$^{+/-}$ zebrafish produced offspring of which approximately 25% had an abnormal phenotype, which was clearly distinguishable from siblings at two days post fertilization. Genotyping confirmed that these phenotypically abnormal fish were *gle1*$^{-/-}$. Both the transgenic mutant and the point mutant fish have identical phenotypes. The mutant fish have a small head and small eyes. At two days post fertilization the mutant fish display cell death in their central nervous system, most clearly visible in the optic tectum, which can be seen when looking through the microscope as a dark mass of cells. Staining by Acridine orange (AO) confirmed that 2dpf *gle1*$^{-/-}$ embryos have significantly more dying cells in their brain and spinal cord than their wild type siblings. Also, the gut and the eyes of *gle1*$^{-/-}$ embryos have marked cell death. (Figure 9)

There is also marked cell death in the hatching gland cells of the *gle1*$^{-/-}$ embryos at two days post fertilization. The hatching gland is a set of cells located on the pericardial membrane of the zebrafish embryo. It appears at the segmentation stage of the embryo (at 14-16 hours post fertilization) and disappears during the pec-fin developmental stage, which starts at 60 hours post fertilization. The hatching gland cells secrete enzymes that degrade the chorion and allow the embryo to hatch. Once the embryo has hatched these cells undergo apoptosis. The difference in the amount of apoptotic hatching gland cells between the mutant and wild type fish could be a

timing issue, which could be further looked into by monitoring the development and apoptosis of hatching gland cells in mutant and wild type fish over time.

By five days post fertilization the *gle1*$^{-/-}$ embryos have severe oedema of the rostral region, a slow heart beat, and significantly reduced movement compared to their wild type siblings. The localization of polyadenylated RNA within the cells of both mutant and wild type zebrafish embryos was assayed by RNA *in situ* hybridization using a probe that binds the polyA-tails of mRNA molecules. DAPI staining was used to visualize the nuclei. As seen in Figure 10, the mRNA within the cells of *gle1*$^{-/-}$ embryos is mostly retained inside or around the nuclei, whereas in wild type embryos, a more diffuse localization throughout the cell is seen.

The retention of mRNA in the cell nucleus would seem like such a grave defect, that one would presume it would be lethal at a very early stage. The images showing a difference in the intracellular localization of mRNA in zebrafish cells are from epithelial cells, suggesting the mRNA retention in the nucleus is not limited to cells of the nervous system. The zebrafish embryo expresses maternal mRNA in the beginning of its development, which could explain why the *gle1*$^{-/-}$ zebrafish are able to survive for so long. The DAPI stain used to visualize the nucleus stains DNA. It would be useful to also stain the nuclear membrane to assess whether the mRNA is actually completely retained inside the nucleus, or partially located around the outer perimeter of the nucleus as well, in which case translation of proteins would be possible. This could also explain why cell death is seen in the nervous system; nerve cells are among the largest cells in vertebrates, and localized translation of mRNAs far away from the nucleus, at the rim of the cell (e.g. the synapse) is critical for their functioning. The retention of mRNA in the proximity of the nucleus would perhaps not be so detrimental to a smaller cell, but large, specialized cells such as neurons, which depend on effective transport of mRNA species to various compartments of the cell, would be vulnerable to such a defect.

The cell death in the central nervous system seems to be more extensive in *gle1*$^{-/-}$ zebrafish than the cell death observed in the human LCCS1 fetuses. This could be due to the differences between the human and zebrafish nervous systems, or difference in the function of gle1 in these two organisms, but also the nature of the LCCS1 disease mutation could explain the difference. The LCCS1 Fin_{Major} mutation is an in-frame insertion that is predicted to result in three extra amino acids in the GLE1 protein. Since there were no GLE1 antibodies available at the time when cell studies could have been conducted with LCCS1 patient cells, we have not assayed GLE1 protein expression in LCCS1 patients. Rather than completely lacking the GLE1 protein, it is possible that patients express a mutant protein, which might still be able to perform the functions of the normal GLE1 protein in the cell to some extent, in which case the complete knockout of *gle1* in zebrafish would not be a

good strategy for studying the LCCS1 disease mechanism. The use of *gle1*$^{-/-}$ zebrafish, however, would allow an experiment in which a rescue of the *gle1*$^{-/-}$ phenotype could be attempted by injection of wild type *gle1* mRNA into fertilized *gle1*$^{-/-}$ zebrafish eggs. A mutant mRNA containing the LCCS1 Fin_{Major} mutation could also be injected into another group of fertilized eggs. This experiment would provide a more accurate assessment of the effects of the LCCS1 Fin_{Major} mutation in zebrafish and determine whether the zebrafish would be a good organism for studying the LCCS1 disease mechanism.

Figure 9. Acridine orange staining of wild type (left) and *gle1*$^{-/-}$ (right) zebrafish. The *gle1*$^{-/-}$ zebrafish have marked cell death in their central nervous system, the gut, and the eyes. They also exhibit increased cell death in the hatching gland cells.

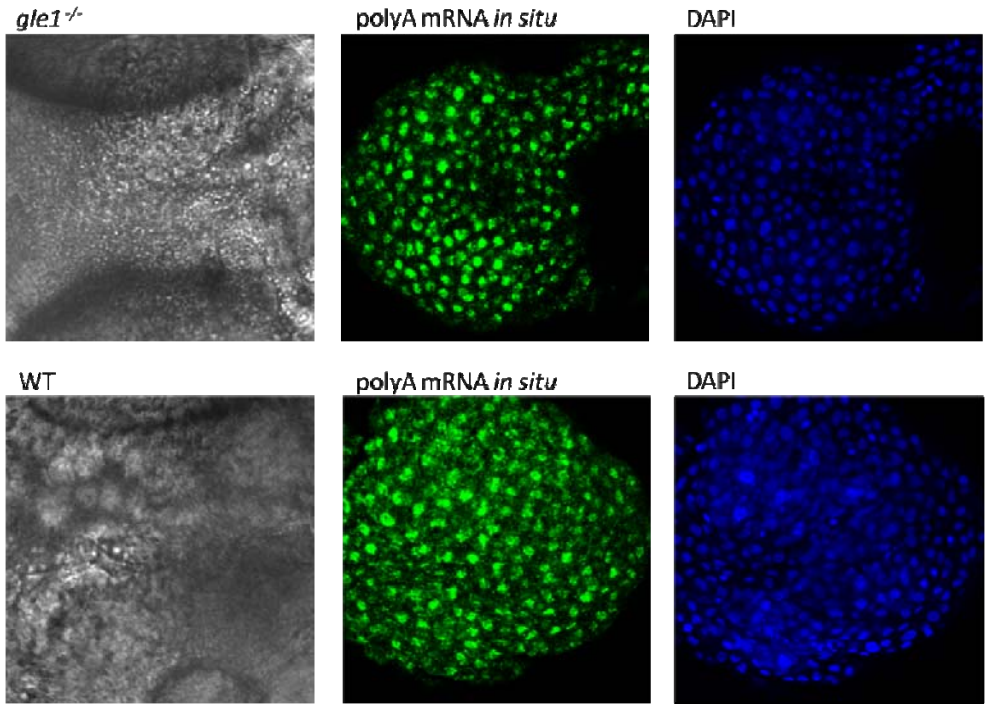


Figure 10. Staining of polyadenylated mRNA in *gle1*$^{-/-}$ (top) and wild type (bottom) zebrafish at 2dpf shows that in mutant cells, the mRNA is mostly retained inside or around the nucleus, whereas in wild type cells, the distribution of mRNA is more even throughout the cell.

5.3 Fetal encasement malformation (II)

As a research group that specializes in deciphering the molecular background of fetal malformation syndromes, we had established close collaboration with the clinicians of the Women's Hospital in Helsinki. The case of one particular family with a unique, very severe fetal malformation recurring in two consecutive pregnancies was brought to our attention. We took up the challenge of investigating this unusual case.

5.3.1 Clinical description of the cases

The family described in this study consists of healthy parents of Finnish origin who, to their knowledge, are not consanguineous. They had five pregnancies. The first pregnancy ended in a spontaneous miscarriage at the end of the first trimester; the fetus was not available for study. In the two consecutive pregnancies, multiple malformations of the fetuses were seen. The fourth and fifth pregnancies resulted in the birth of normal infants.

An ultrasonogram examination carried out during the 14^{th} and 13^{th} weeks of the second and third pregnancies, respectively, displayed identical, abnormal findings. In both cases, an abnormal cyst in the cranial part of the fetus, malformations of the craniofacial area, and an omphalocele were seen. The limbs were immotile and visible only as small buds. Based on these ultrasonogram findings, the pregnancies were terminated at the 15^{th} and 14^{th} weeks of gestation, respectively. The autopsy revealed that the extremities of the fetuses were actually encased by the abnormal, membrane-like, transparent skin. Both fetuses had a bifid orifice in the middle of the face, and small eyes situated on either side of this formation. The mouth was just a depression covered with skin. In addition to these findings, the first fetus had a defect in the diaphragm, a cardiac defect (Fallot tetralogy), a horseshoe kidney, underdeveloped skull bones, and a lobulation defect in both lungs with four lobes in the right lung and three lobes in the left lung. The second fetus also had a lobulation defect with three lung lobes in each lung, but the other inner organs appeared normal. An X-ray of the first case revealed that the bones of the spine, thorax, lumbar area, base of the skull, face, and the extremities existed, but were hypoplastic. There were a normal number of vertebrae and ribs, but the spine was scoliotic. Histological examination on bone structure revealed a normal growth plate and no features of chondrodystrophy (a congenital hamartia of cartilage). There were no specific histological changes visible in the internal organs. However, histological examination on skin sections of the extremities showed a thin, membrane-like epidermis with only two to three cell layers (Original publication II, Figure 2). Primitive hair follicles were also found. Within the bony structures of the head, there

was an island of squamous epithelium, which probably represents an unopened mouth. Both fetuses had a normal, female karyotype (46, XX), which rules out large chromosomal rearrengements. Pictures of the affected fetuses can be found in Figure 1 of Original publication II.

5.3.2 Genealogical investigations

The recurrence of this unusual phenotype in one family suggested autosomal recessive inheritance, so we undertook genealogical investigations in order to find out if the parents were distantly related. We traced ancestors back to 1850 from Finnish Population Register Centre and local church registers. For earlier periods, we scrutinized microfiche copies of local church records available in the National Archives of Finland. Based on this analysis, the parents share a common ancestor; they are both the descendants of a couple that lived in Ristijärvi (Figure 11) during the 18th century. From the father's side, this consanguinity dates back nine generations, and from the mother's side, seven generations (Figure 11). This consanguinity reflects the typical population substructure of Finland, and supports the theory of autosomal recessive inheritance.

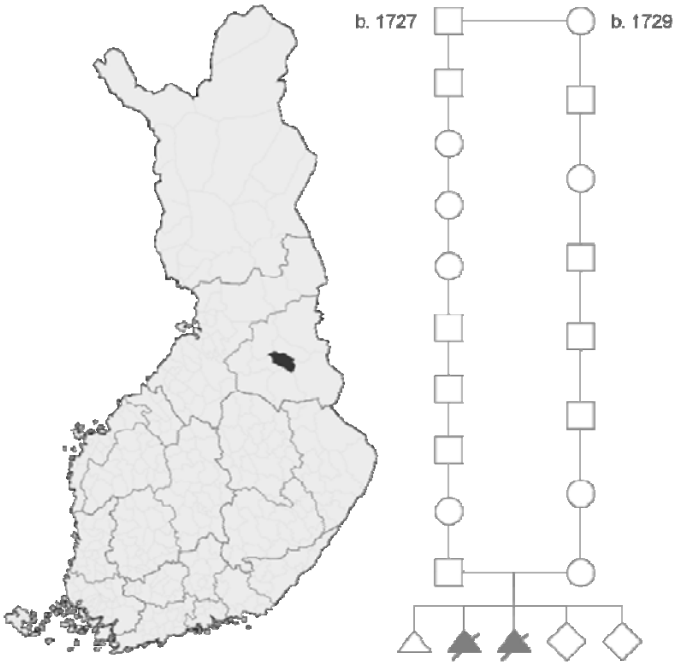


Figure 11. Genealogical investigations suggest that the couple share a common ancestor in 18th century Ristijärvi (marked in black on the map)

5.3.3 Genome-wide gene expression array analysis

We established genome-wide gene expression arrays from fetal fibroblast cell lines of the two cases and three healthy controls aborted for social reasons using the Affymetrix platform in order to gain knowledge about the molecular pathways behind the encasement malformation. The analysis of expression data produced a list of 132 transcripts that were differentially expressed in cases and controls (a list of the differentially expressed genes can be found in the Supplementary table 1 of Original publication II).

Methods of gene expression array data analysis rely on statistics to point out the most significant result. We performed the standard statistical analyses, but in addition to this, also looked further into the differentially expressed genes in order to gain insights into the biology behind the statistics. Since animal models have successfully been utilized in the search for disease causing mutations in humans (Chow et al. 2007), we searched the Mouse Genome Database (Bult et al. 2008) for murine knockouts of the genes that were downregulated in the affected fetuses.

Interestingly, mice lacking the conserved helix-loop-helix ubiquitous kinase *Chuk* (also known as *Ikk1* and *Ikkα*) bore a striking phenotypic resemblance to our cases (for a detailed description of the *Chuk* $^{-/-}$ *mouse* phenotype, see section 5.3.6). The *CHUK* transcript was downregulated 8.5-fold in our cases, making it an excellent candidate gene for the syndrome. The comparison of human and mouse CHUK proteins by Basic Local Alignment Search Tool shows a 95% similarity, which suggests this protein has an analogous, conserved function in both species and therefore the impairment of human CHUK might result in a phenotype parallel to that of the *Chuk* knockout mouse. It is important to note that the difference in the expression of the *CHUK* transcript between cases and controls was less significant than that of many other transcripts, but combining existing biological information with the results of the statistical analysis clearly made *CHUK* the single most attractive candidate gene.

5.3.4 Analysis of the CHUK gene and CHUK protein

To search for the causative mutation, we amplified the coding region of *CHUK* (NM_001278) from the cDNA of the cases and two control samples. Sequence analysis of *CHUK* revealed that both affected fetuses were homozygous for a C to T substitution (c.1264C>T) in exon 12 of *CHUK*. This mutation was also observed in the genomic DNA of the fetuses. Sequencing of the genomic DNA of both parents showed that both of them are heterozygous carriers of this mutation. We then screened one hundred ancestrally matched controls for the mutation, and found no heterozygote carriers or homozygotes. The *CHUK* gene consists of 21 exons, and encodes a protein of 745 amino acids. The 1264C>T mutation changes the glutamine at position 422 to a stop codon (p.Gln422X), which predicts a truncated polypeptide of 421 amino acids, assuming that the mutant messenger RNA is not degraded (Figure 12). To assay CHUK protein expression, we used immunoprecipitation followed by western blot analysis of cell lysates made from fibroblasts of the fetal skin. We used an antibody that binds to the N-terminal part of CHUK to establish whether affected fetuses express a truncated protein product and observed no bands in lanes containing samples derived from the cases, suggesting that mutant *CHUK* RNA is degraded via the nonsense-mediated decay mechanism and mutant protein is not expressed. (Original publication II, Figure 3)

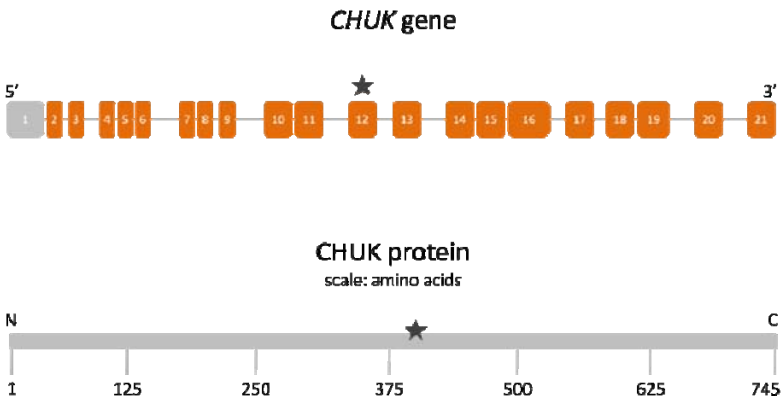


Figure 12. The *CHUK* gene and the CHUK protein. The mutation in exon 12 and amino acid position 422 is marked with a star.

Nonsense-mediated mRNA decay (NMD) is a pathway that targets mRNAs that contain premature termination codons (PTCs) and marks them for degradation (Baker and Parker 2004, Holbrook et al. 2004, Maquat 2004, Conti and Izaurralde 2005, Fasken and Corbett 2005, Lejeune and Maquat 2005, Wilkinson 2005, Yamashita et al. 2005, Chang et al. 2007). This pathway is important for the maintenance of cellular integrity, since PTC-containing messages would produce truncated proteins with potentially deleterious gain-of-function or dominant-negative activity, if they were allowed to be translated. NMD is a highly conserved pathway that exists in all eukaryotes (Culbertson 1999).

The core NMD machinery is made up of three trans-acting factors, called up-frameshift (UPF) proteins, the UPF1, UPF2, and UPF3 (Leeds et al. 1992, Cui et al. 1995, Lee and Culbertson 1995). UPF1 is recruited to mRNAs upon recognition of stop codons by the translation apparatus (Czaplinski 1998, Kashima et al. 2006). The interaction of UPF1 with UPF2 and UPF3 triggers rapid decay of PTC-bearing mRNAs. In mammalian cells, UPF2 and UPF3 are part of the exon-junction complex (EJC), a large complex of proteins that is deposited on mRNAs at exon-exon junctions during RNA splicing in the nucleus. The EJC is thought to play an important role in distinguishing premature stop codons from legitimate ones and thus determining which mRNAs are marked for NMD (Chang et al. 2007). In addition, NMD requires factors that regulate UPF1 phosphorylation. SMG-1, SMG-5, SMG-6, and SMG-7 control the phosphorylation status of UPF1 (Wilkinson 2003). All four SMGs are required for NMD, since mutations in any one of these components inhibits NMD (Chang et al. 2007).

Mammalian NMD seems to occur as a result of PTC recognition during a proofreading round of translation, since the substrate for this proofreading round of translation has a different mRNP composition than subsequent rounds of translation that generate large amounts of protein (Chang et al. 2007). Upon translation termination, release factors recruit UPF1 and the UPF1 kinase SMG-1 to form the SURF complex, a transient complex that quickly interacts with the EJC through UPF2. This SURF-EJC interaction defines the stop codon as premature. Once phosphorylated, UPF1 attracts the phosphoserine-binding domain proteins SMG-5, SMG-6, and SMG-7 that promote the dephosphorylation of UPF1. SMG-7 is thought to be the terminal effector of NMD because it accumulates in P-bodies, cytoplasmic sites of mRNA decay. SMG-7 also elicits rapid decay when tethered to any position within an mRNA, which is an unique characteristic among NMD proteins. Recent studies suggest that mammalian NMD is not a single pathway, but has several branches, some of which may use alternative EJCs and others that may operate independent of EJCs (Chang et al. 2007).

5.3.5 CHUK and NF-κB

The conserved helix-loop-helix ubiquitous kinase CHUK is also known as IκB kinase α (IKKα), and it is a subunit of the multiprotein complex IκB kinase (IKK). IKK has two catalytic subunits, CHUK and IKKβ, and a regulatory subunit IKKγ (NEMO). In response to proinflammatory stimuli, the IKK complex phosphorylates IκB proteins, which in turn lead to IκB degradation. IκB inhibits NF-κB/Rel transcription factors by blocking their nuclear localization sequence. When IκB function is abolished, NF-κBs translocate to the nucleus, where they regulate target genes (Hu et al. 1999). (Figure 13)

CHUK and IKKβ share over 50% amino acid identity, and they both phosphorylate IκB proteins at serines, but it has been shown that CHUK is not required for NF-κB activation (Hu et al. 2001). Instead, it regulates epidermal development and skeletal morphogenesis in an NF-κB independent manner (Hu et al. 1999, Li et al. 1999, Takeda et al. 1999, Sil et al. 2004).

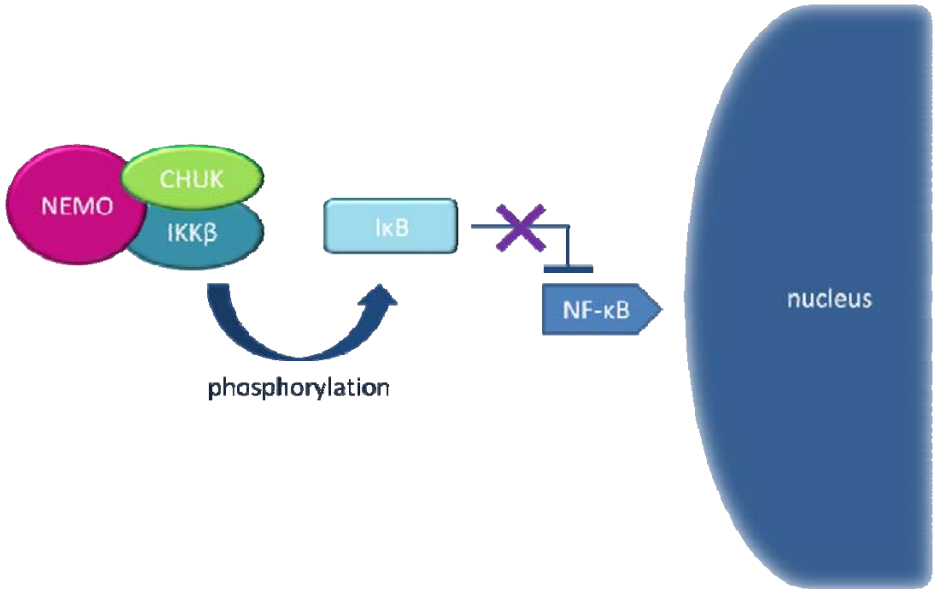


Figure 13. CHUK is part of a multiprotein complex IκB kinase that regulates NF-κB transcription factors.

5.3.6 *Chuk* $^{-/-}$ mice

Mice that are deficient in Chuk (*Chuk* $^{-/-}$ mice) have been independently generated by three different research groups (Hu et al. 1999, Li et al. 1999, Takeda et al. 1999). *Chuk* $^{-/-}$ mice are carried to term, but die shortly after birth. The pups have multiple morphogenetic defects. Their skin is unusually shiny, thick, and adhesive, and it encases the limbs, which appear externally only as rudimentary protrusions. Limb bones, however, are close to normal shape, and of almost normal length, but they are tightly folded under the skin. The tail is also rolled up under the skin. The pups have a truncated snout and no external ears, and multiple skeletal defects. Mutant fetuses also have an omphalocele, and the placentae are severely congested with bulging vessels and blood sinuses on the maternal surface and normal fetal surface. Histological staining of skin sections of the *Chuk* $^{-/-}$ mice with hematoxylin and eosin shows hyperplasia of the suprabasal layer (*stratum spinosum*), and lack of the two outer layers of the epidermis, *stratum granulosum* and *stratum corneum*. The increased thickness of the suprabasal layer of the mutant epidermis results from hyperproliferation of the keratinocytes and their inability to differentiate (Hu et al. 1999). In mutant pups, the esophagus, an organ whose inside is also covered in keratinocytes, is closed up. No obvious abnormalities of the heart, lungs, liver, kidney, spleen, brain, or spinal cord are seen at autopsy. (Hu et al. 1999, Li et al. 1999, Takeda et al. 1999)

The phenotypic similarity between the *Chuk* $^{-/-}$ mice and human fetuses lacking CHUK is remarkable. Both have limbs that are encased under the skin, but limb bones are close to normal shape and length. However, both the *Chuk* $^{-/-}$ mice and the human fetuses have other skeletal defects, including the absence of some of the skull bones (Hu et al. 1999). *Chuk* $^{-/-}$ mice have fused vertebrae, a small thoracic cage, and a cleft palate (Hu et al. 1999, Li et al. 1999). This is interesting, since one of the genes downregulated in our affected fetuses is the Cleft lip and palate associated transmemmbrane protein *CLPTM1*. A balanced translocation disrupting the *CLPTM1* gene was discovered in a family with cleft lip and palate, but screening of the *CLPTM1* gene in other patients with nonsyndromic cleft lip and palate failed to identify causative pathogenic mutations (Yoshiura et al. 1998). The human fetuses lacking CHUK have a normal number of vertebrae and ribs, but the spine is scoliotic. However, craniofacial malformations seem to be more severe in human fetuses than in *Chuk* $^{-/-}$ mice, suggesting that CHUK has a substantial role in human craniofacial morphogenesis, although the precise mechanism by which CHUK is involved in the development of craniofacial structures still remains to be discovered. The cyst seen in the ultrasound examination of the human fetuses could be the esophagus, since in the *Chuk* $^{-/-}$ mice, the esophagus is closed up due to hyperproliferation of keratinocytes in the esophageal epithelium.

5.3.7 Pathway analysis of the gene expression array data

To investigate the cellular pathways and processes disturbed by the absence of CHUK in our affected fetuses, we analyzed the expression data further using the WebGestalt program (Zhang et al. 2005). WebGestalt is a WEB-based GEne SeT AnaLysis Toolkit that processes gene lists, and groups differentially expressed genes into functional categories. The WebGestalt program recognized 91 of the 132 transcripts that were differentially expressed in our cases and controls. We grouped the differentially expressed genes in three different ways, according to:

1. Biological process
2. Molecular function
3. Cellular component

Grouping according to biological process showed that in the affected fetuses, genes that regulate ossification, establishment of protein localization, cell motility, phosphate transport, and the I-κB kinase/NF-κB cascade were significantly different from controls. When differentially expressed transcripts in cases and controls were classified by molecular function, genes related to actin filament binding, protein phosphatase inhibitor activity and the extracellular matrix stood out. When over-represented genes were classified according to cellular component, those encoding proteins of the extracellular region and matrix (ECM), cytoskeleton, and ER-Golgi intermediate compartment were most highly ranked. (Original publication II, Supplementary Table 2 and Supplementary Figure 1)

5.3.8 MMP-14

The extracellular matrix category included a large number of genes downregulated in the affected fetuses. One of these genes encodes the matrix metallopeptidase (MMP-14), essential for skeletal development (Zhou et al. 2000) and formation of blood vessels (Davis et al. 2007). We performed a western blot analysis of the fetal fibroblast cell lysates to assay for MMP-14 protein expression and, unexpectedly, observed protein expression in the fibroblasts of affected fetuses but not those of age-matched controls (data not shown). This could be explained, for example, by a negative feedback loop mechanism. Given the complexity of posttranscriptional mRNA processing discussed in section 5.2.4, it is known that mRNA levels in the cell do not necessarily correspond with protein expression levels (Moore 2005). Mice deficient in MMP-14 exhibit impaired endochondral ossification and angiogenesis (Zhou et al. 2000). Interestingly, also keratinocyte-specific Chuk knockout mice displayed increased vascularization in their paws (Gareus et al. 2007), suggesting a link between the Chuk and Mmp14 proteins.

5.3.9 CHUK and NF-κB signalling in the development of ectodermally derived structures

Based on phenotypic observations of *Chuk* $^{-/-}$ mice it was concluded that Chuk regulates epidermal development and skeletal morphogenesis in an NF-κB independent manner. Keratinocyte-specific Chuk knockout mice, however, exhibit a differentiated epidermis with normal epidermal layers, suggesting that the ability of Chuk to induce epidermal differentiation is not keratinocyte-autonomous. Despite a normally stratified epidermis, the keratinocyte-specific Chuk knockout mice have defects in stratum corneum lipid composition and in epidermal tight junctions, which leads to impaired epidermal-barrier function and increased transepidermal water loss. These defects are caused by the deregulation of retinoic acid target genes that encode key lipid modifying enzymes and tight junction proteins in keratinocytes. (Gareus et al. 2007)

Ohazama and colleagues determined that Chuk is expressed in the murine tooth epithelium and has an essential role in incisor and molar tooth development. Molar teeth in Chuk deficient mice, as well as in mice expressing a super-repressor of the NF-κB pathway, had abnormal cusp morphology, indicating that Chuk is involved in cusp formation through the NF-κB pathway. Incisors, however, had an earlier-onset and more severe phenotype, where tooth buds evaginated into the developing oral cavity instead of invaginating into the underlying mesenchyme, as supposed to. A similar evagination of epithelium was also observed in whisker follicles of Chuk mutant animals. Unlike cusp morphogenesis, the NF-κB pathway was not involved in the invagination of incisor tooth epithelium. (Ohazama et al. 2004)

NF-κB signalling is also involved in other ectodermal disorders. Mutations in the *EDA*, *EDAR*, and *EDARADD* genes that encode activators of the NF-κB pathway, cause hypohidrotic ectodermal dysplasia in humans and mice (Kere et al. 1996, Ferguson et al. 1997, Srivastava et al. 1997, Headon and Overbeek 1999, Monreal et al. 1999, Headon et al. 2001). This disorder causes defects in the development of cutaneous structures and is characterized by sparse hair, a lack of sweat glands and malformation of teeth. Although these proteins that are crucial for normal hair follicle development directly regulate NF-κB signalling, primitive hair follicles were still seen in our fetuses lacking CHUK, suggesting that even in the absence of CHUK, there is sufficient NF-κB signalling for hair follicles to be formed.

5.3.10 CHUK, keratinocyte differentiation and skin cancer

Chuk deficiency results in the hyperproliferation of keratinocytes, which can be seen in the abnormal epidermal structure of *Chuk* $^{-/-}$ mice that completely lack the granular and cornified layers of the epidermis (Hu et al. 1999, Li et al. 1999, Takeda et al. 1999). Cultured *Chuk* $^{-/-}$ keratinocytes do not express the terminal differentiation marker filaggrin, and they continue to proliferate even when subjected to differentiation-inducing stimuli (Hu et al. 2001).

Squamous cell carcinoma (SCC), a cancer derived from squamous epithelia of the skin, head and neck tissues, is the second most common skin cancer in Caucasians (Johnson et al. 1992). Mutations in exon 15 of *CHUK*, which resulted in reduced *CHUK* expression, were described in a few high-grade and poorly differentiated human SCCs of the skin (Liu et al. 2006). Epigenetic silencing of the *CHUK* locus is seen in close to 30% of invasive oral SCCs (Maeda et al. 2007). In mice, suprabasal overexpression of CHUK results in increased epidermal differentiation and reduced keratinocyte proliferation, and inhibits chemically induced SCC formation and progression (Liu et al. 2006). *Chuk* $^{+/-}$ mice developed two times more papillomas and 11 times more carcinomas than *Chuk* $^{+/+}$ mice, and loss of the wild type *Chuk* allele was seen in some papillomas and most carcinomas (Park et al. 2007). Thus it can be concluded that CHUK acts a tumor suppressor in the epidermis by controlling keratinocyte proliferation. (Descargues et al. 2008) However, the role of Chuk in cancer seems to be more complex, since studies in mice with mutations in *Chuk* and *Ikkβ* suggest that (wild type) Chuk promotes metastasis, and Ikkβ promotes tumorigenesis (Karin 2008).

5.3.11 Cocoon syndrome

In 1987, Stevenson and colleagues described a “cocoon fetus” born to consanguineous parents of Palestinian descent. The male fetus was born at 27 weeks gestation, and his extremities were folded and fused to the trunk, although they appeared to be of normal lenght. In addition, the fetus had microcephaly, absent pinnae, protruding eyes, a protruding tongue, and the mandible was absent. The nose was a sharp, beak-like structure. Histological examination of skin showed a thinned dermis with no sweat glands or hair follicles, and a thinned epidermis with hyperkeratosis. The brain was poorly developed with widely separated cerebral hemispheres that lacked the usual morphological landmarks. (Stevenson et al. 1987)

We decided to name our novel fetal encasement malformation Cocoon syndrome because of the cocoon-like appearance of the fetuses, and according to the ”cocoon fetus” reported by Stevenson and colleagues. The craniofacial malformations seen in Stevenson’s case are different from the defects seen in our fetuses, and the structure of the skin is also different, since our cases had hair follicles. Also, contrary to the hyperkeratosis of the *stratum corneum* observed in Stevenson’s case, the deficiency of Chuk results in the absence of the *stratum corneum* and hyperproliferation of basal keratinocytes.

Following the publication of Original publication II, Cocoon syndrome was assigned with a MIM number (MIM 613630).

6 Conclusions and future prospects

This study identified the causative mutations underlying fetal motoneuron diseases LCCS1 (MIM 253310) and LAAHD (MIM 611890). In addition, we described a novel clinical entity, the Cocoon syndrome (MIM 613630), and its causative mutation. Based on the results of this study, DNA based diagnostics of these diseases is now possible, and in the case of LCCS1 and LAAHD, already in practice at the Helsinki University Hospital Laboratory (HUSLAB).

The study provided significant new information about the molecular mechanisms underlying fetal motor neuron disease by showing that *GLE1*, a gene encoding a multifunctional protein involved in posttranscriptional mRNA processing, is mutated in LCCS1 and LAAHD. This observation, along with genetic discoveries in other motor neuron diseases such as ALS and SMA, highlight the significance of mRNA processing in motor neuron disease. Studies on mutant *gle1*$^{-/-}$ zebrafish provided encouraging evidence that LCCS1 pathogenesis could be studied in a zebrafish model and laid ground for further experiments. The molecular pathway connecting the LCCS1, LCCS2, and LCCS3 disease genes would be an interesting line of research. Transgenic mouse models defective in these genes could be employed to further evaluate and compare the cellular and developmental consequences of the loss of these molecules, as well as their relationship and interactions with one another. Studies on Cocoon syndrome suggest that the conserved helix-loop-helix ubiquitous kinase CHUK influences both human and murine development in a similar manner. However, the craniofacial abnormalities in human fetuses lacking CHUK are more severe than defects seen in the *Chuk*$^{-/-}$ mice, suggesting an important role for CHUK in human craniofacial morphogenesis.

During the course of this study, the field of human genetics has come a long way. The completion of the Human Genome Project, the current advances in sequencing technologies, as well as the increasing availability of web-based resources have shifted the focus of human genetic studies from in vitro to in silico. The challenge is no longer in producing sequence data, but in analyzing this vast amount of information, and understanding the molecular and cellular events that lead to disease. The data collected from studies on model organisms has been extremely valuable to this study. The use of animal and cell models is also vital in further studies of the molecular mechanisms behind human disease.

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